

AFLATOXIN CONTAMINATION, HUMAN EXPOSURE, AND
OPPORTUNITIES IN THE HAITIAN PEANUT VALUE CHAIN

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AFLATOXIN CONTAMINATION, HUMAN EXPOSURE, AND OPPORTUNITIES IN THE HAITIAN PEANUT VALUE CHAIN

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Aflatoxins (AFs) are carcinogenic, secondary metabolites of the molds *Aspergillus flavus* and *A. parasiticus* and contaminate grains and oil seeds, particularly maize and peanuts. The risk of AF contamination exists along entire peanut and maize value chains where climate is suitable, typically among tropical and sub-tropical areas. A value chain is the complete array of activities required to produce and deliver a commodity to consumers, including intermediary processing and storage. In the Caribbean country of Haiti, capacity to prevent human exposure to toxic substances, both natural and xenobiotic, is limited. The present dissertation examined: 1) constraints to AF control among peanut farmers in Haiti; 2) contamination in maize and peanut products, as well as edible oil production as an alternative process to salvage a safe, value-added product from contaminated peanuts; and 3) human exposure to AF among urban and rural communities. My formative survey of 109 peanut farmers in the North and North East Departments underscored a constrained agronomic and socio-economic context under which farmers lacked access to credit, irrigation, pesticides, and other agricultural inputs necessary to improve production and food safety. Twenty-three farmers were familiar with AFs or the fungi that produce them. AF analysis of foods sampled from 2012 to 2013 showed that samples of peanuts (3/21), peanut butter (30/32), and maize (1/30) exceeded the US Food and

Drug Administration limit for AFs in human foods (20 µg total AFs/kg). AF concentration was greatest in peanut butters (median of 137 µg/kg and maximum of 2720 µg/kg). Experiments to determine residual AFs in oil pressed from contaminated peanuts showed that 5% the AF concentration of the original kernels remained in the oil. Ethanol extraction further reduced AF concentration to 10% that of the pre-extraction oil. Among Haitians screened for AF biomarkers, detection of AF-lysine in circulating blood albumin was associated with recalled frequency of peanut consumption ($p=0.0486$) but not maize consumption ($p=0.2032$). Detection of urinary AFM1 was more significantly associated with consumption of peanuts the day prior to study participation ($p<0.001$) than maize ($p=0.105$). The studies of human exposure and food contamination reported herein augur the need for suitable and timely interventions along peanut and maize value chains in Haiti.

BIOGRAPHICAL SKETCH

Jeremy Schwartzbord was born and raised in the San Francisco bay area. Since his *bar mitzvah* in 1998, Jeremy has tended to sate the curiosities of his intellect in circuitous ways, often against better judgment. He cultivated interest for the biological sciences in the Sierra Nevada mountains: backpacking, deep snow camping, and hiking with Boy Scout Troop 301. After attaining his Eagle Scout award and finishing high school, he pursued a Bachelors of Science in Conservation and Resource Studies with a minor in Forestry at the University of California, Berkeley. The breadth of opportunity at Cal proved apt to Jeremy's eclectic and fledgling interests, such as foreign languages, theatrical improvisation, and biological sciences, and his undergraduate education included a yearlong study in Chile with the UC Education Abroad Program. Following graduation from Cal, Jeremy did an internship with the Mexican non-profit organization *Red de Acción sobre Plaguicidas y sus Alternativas en México* (Pesticide Action Network of Mexico), an experience that kindled his interest in toxicology. Jeremy was awarded a Fulbright English Teaching Assistantship, which he carried out in Venezuela in 2008. In 2010, he had the opportunity to pursue doctoral studies at Cornell University in the Field of Environmental Toxicology under the mentorship of Dr. Dan Brown. Concurrent with his Cornell matriculation, Jeremy knew little of aflatoxins, Haitian Creole, or appropriate clothing to match Ithaca's wintry gusts. In the meantime, he learned a few things.

To my parents, Aaron & Michael, and Stephanie for your copious love and support.

To many others, in Cap Haitien and Ithaca, who offered patience and a guiding hand along this path.

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The present dissertation is a product of ample guidance, collaboration, support, and good humor that others offered me throughout five years and four research trips to Haiti. Cornell University's Bradfield Research Award made possible my first two visits to the city of Cap Haitien in 2011, and I extend my appreciation to the Cornell Department of Animal Science and the Field of Toxicology, which both made available support for part of my stipend. My third trip to Haiti took place thanks to generous funding from the US Agency for International Development Title XII Peanut Collaborative Research Support Program. USAID PCRSP and the Peanut Mycotoxin Innovation Lab supported laboratory materials needed in Ithaca. Purdue University's US Borlaug Global Fellows in Food Security Program made my fourth and longest research trip to Haiti possible. As a Borlaug Fellow, I was very fortunate to have Dr. Jef Leroy of the International Food Policy Research Institute as a mentor, particularly as I composed the final chapters of this manuscript. Furthermore I am grateful to the Hyman and Miriam Lockwood Scholarship for the Max and Ida Lechhook Fellowship, which was a critical source of my stipend support; I thank Dr. Richard Lockwood for his encouragement throughout my PhD studies.

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Present and past staff at MFK, including Executive Director Dr. Pat Wolff and former head of agricultural programming Jamie Rhoads, both of whom were tremendous, albeit informal, mentors to me. Pat's tenacity, relentless energy, and 25+ years of experience in Haiti were inspiring; her recurrent "A exam"-style questioning and helpful advice tempered my logistical planning enough to withstand the vagaries of conducting research in Haiti. Special thanks are due to Jamie Rhoads, who first retrieved me from a dusty bus station in Cap Haitien and ever since has expounded his vast knowledge of peanuts and Haiti to me.

The lab of JS Wang, then at Texas Tech University, analyzed blood samples obtained in Port-au-Prince for our report of circulating aflatoxin-lysine adducts in Haitians consuming maize and peanuts. Rebecca Heidkamp interviewed clients who donated a blood sample, and she salvaged data records from the health center GHESKIO following the 2010 earthquake that shook Port-au-Prince.

Back in Ithaca, I wish to thank Joanne Parsons and Barbara Jones for their administrative support and Suzane Pelton for laboratory guidance. A small army of students was essential to expediting my lab work and included Michael Komrowski, Kelsey Smith, Julie McDonnough, Mana Okudaira, and Kaitlin Smith. Long days in the sanatorium-evoking corridors of Morrison Hall were possible because of commiseration with fellow graduate students: Katherine Churchill, Keenan McRoberts, Matt Barcus, Maureen Valentine, Natasha Petifor and Meghan Filbert.

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LIST OF ABBREVIATIONS

AF: aflatoxin

AFB1: aflatoxin B1

AFB2: aflatoxin B2

AFG1: aflatoxin G1

AFG2: aflatoxin G2

AFM1: aflatoxin M1

AF-Gua: 2,3-dihydro-2-(N7-guanyl)-3-hydroxyaflatoxin B1

AP: agricultural programming

CL: *clarin*

ELISA: enzyme-linked immuno-sorbent assay

FAO: Food and Agriculture Organization of the United Nations

FDA: US Food and Drug Administration

GHS: Reduced glutathione

HA: high aflatoxin oil

HCC: hepatocellular carcinoma

HBV: hepatitis B virus

HPLC: high pressure liquid chromatography

LA: low aflatoxin oil

LOD: limit of detection

LOQ: limit of quantitation

MFK: Meds & Food for Kids

RUF: ready-to-use foods

PART 1

CHAPTER 1: PROBLEM STATEMENT AND SUMMARY OF STUDIES

Problem Statement

Aflatoxins are secondary metabolites of the fungi *Aspergillus flavus* and *A. parasiticus*, which infect staple grains and oil seeds in places where climate is favorable to growth and toxin production. Thus, aflatoxins are prevalent in tropical and sub-tropical countries, particularly where food safety measures are limited among agricultural producers and food processors. Maize and peanut products are especially susceptible to aflatoxin contamination.

Aflatoxin B1, the most prevalent and toxic of aflatoxins, is a potent carcinogen to the liver, and up to 28% of worldwide hepatocellular carcinoma cases are attributed to chronic aflatoxin exposure (International Agency for Research on Cancer, 2010; Liu & Wu, 2010). Aflatoxin exposure is associated with other adverse health effects, such as stunting and immune-dysfunction in children (Gong et al., 2003; Turner, Moore, Hall, Prentice, & Wild, 2003).

Globally, aflatoxin threatens food safety throughout many stages of maize and peanut value chains, because those commodities are susceptible to aflatoxin contamination prior to and after harvest. I borrow the concept of a value chain from Kaplinsky (2004) who defines it as “the full range of activities that are required to bring a product or service from conception, through the intermediary phases of production (involving a combination of physical transformations and the input of various producer services), delivery to final consumers, and final disposal after use” (Kaplinsky, 2004). His concept of

value chain is inherently general, allowing for complexities of different food production systems.

Prior to 2009, few published studies had examined aflatoxin contamination, exposure, or control in Haiti, an island nation of approximately 10 million people in the Caribbean region. Two investigations had shown evidence of elevated aflatoxin contamination in Haitian maize and peanut products (Castor, Mirocha, & Chang, 1987; Filbert & Brown, 2012). Furthermore, Filbert and Brown (2012) offered a proof of principle demonstrating feasibility of peanut-based fuel patties as an alternative use of peanuts to redirect highly contaminated kernels from direct human consumption in Haiti. No peer-reviewed studies had examined the prevalence of urinary or blood biomarkers to characterize human aflatoxin exposure, the capacity of farmers to manage contamination in their crops, and the efficacy of other alternative uses for contaminated foods in Haiti. Thirty years had passed since the most recent published survey of aflatoxin contamination in maize. A broad examination of aflatoxin contamination in peanut and maize products, exposure among people ingesting those foods, and capacity to manage aflatoxin contamination along food value chains in Haiti had been long overdue.

Summary of Studies

The present dissertation offers evidence of aflatoxin contamination and dietary exposure in Haiti, and the underlying context and systemic constraints of peanut production in Haiti are illustrated. Following chapter 2, this dissertation is a compilation of manuscripts prepared in the style of the journal that each was or will be submitted to.

Part 1 (Chapters 1, 2 and 3) acquaints the reader not only to the toxic agent starring in this dissertation, but also the toxicological context of Haiti. Chapter 2 provides an overview of aflatoxin toxicology, including occurrence, exposure, distribution in the human body, elimination, activity at target site, mechanism of action, and regulation. Survey of those topics is brief and directs the reader to authoritative reviews published elsewhere. Less comprehensively described in the literature is the landscape of past and present toxic threats in Haiti, the focus of chapter 3. That text, "Haiti's food and drinking water: a review of toxicological health risks," was published in the journal *Clinical Toxicology* (J R Schwartzbord, Emmanuel, & Brown, 2013) and includes a section "Heavy metal contamination," which was originally composed by Prof. Evens Emmanuel.

Part 2 (Chapters 4 and 5) focuses on the socio-economic context of the peanut value chain in Haiti, includes a survey of contamination conducted from 2012 to 2013, and examines the safety and efficacy of edible oil production from contaminated peanuts. Chapter 4, "Aflatoxin control and livelihoods among peanuts farmers in north east Haiti: A formative survey," characterizes the socio-economic context of peanut farming in Haiti and was conducted among peanut farmers of the North East Department. In partnership with Lora Iannotti and Colleen Smith of Washington University in St. Louis and the non-governmental organization Meds & Food for Kids, our survey of 109 peanut farmers revealed that a majority of farmers we interviewed were unaware of aflatoxin. Most had limited access to agricultural technologies, such as improved peanut varieties, irrigation, fungicides, and mechanized equipment.

Whereas Chapter 4 illustrates a system where farmers have limited means of maintaining food safety throughout production, the following chapter draws attention to the consequent food contamination. Chapter 5, “Monitoring of aflatoxin contamination in Haitian maize and peanut products and an ethanol extraction process to reduce contamination in edible oil” (Jeremy R. Schwartzbord & Brown, 2015) shows evidence of alarmingly elevated aflatoxin concentration in Haitian peanut butters and reports one alternative process to produce a value-added product that diverts contaminated kernels out of the peanut value chain.

Part 3 (Chapters 6, 7 and 8) examines dietary sources of aflatoxin exposure in Haiti by measurement of blood and urinary biomarkers among participants recruited in urban and rural communities. Chapter 6 reports evidence of aflatoxin-lysine adducts (the Pronase digestion products of circulating aflatoxin covalently bound to blood albumin) in Haitian patients ingesting peanuts and maize. That work was conducted in collaboration with the laboratory of J.S. Wang of the University of Georgia and published by the *Journal of Hunger and Environmental Nutrition* (J R Schwartzbord et al., 2014). Chapter 7 presents initial analysis of urinary aflatoxin M1 (AFM1), using high pressure liquid chromatography (HPLC) and enzyme-linked immuno-sorbent assay (ELISA), among human subjects recruited at a hospital in Port-au-Prince. Chapter 7 was prepared per the guidelines of the journal *Biomarkers* and submitted as a technical brief. Chapter 8 reports urinary AFM1 measured by HPLC for 367 patients recruited in Port-au-Prince and Quartier Morin, a rural municipality 8 km away from the city of Cap Haitien in the North East Department. Recruitment occurred during 2012 and 2013 in Port-au-Prince

and Quartier Morin, respectively. Chapters 6 and 8 complement biomarker data with patients' recalled consumption of peanuts, maize, dairy products, and other animal-sourced foods. Overall, the present dissertation investigates the systemic insult of aflatoxin contamination and exposure in Haiti, viewing that challenge through lenses of agricultural development, food safety, and public health.

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CHAPTER 2: OVERVIEW OF AFLATOXIN TOXICOLOGY: OCCURRENCE, EXPOSURE, DISTRIBUTION, ELIMINATION, TARGET SITE, MECHANISM OF ACTION AND REGULATION

Introduction

The first known case of aflatoxin (AF) intoxication took place during 1960 when 100,000 turkeys unexpectedly died in the United Kingdom. Dubbed “Turkey X Disease,” autopsies of the intoxicated animals revealed damage to the liver, kidney and spleen. In 1962, British researchers attributed Turkey X Disease to the fungal species *Aspergillus flavus* and named the causative agent *Aflatoxin*. During the period of more than fifty years since its identification, AF has been shown to be a causative agent of liver cancer to humans (International Agency for Research on Cancer, 2010) and is implicated in up to 28% of cases of liver cancer worldwide (Liu & Wu, 2010), the fifth- and seventh-most common cancer in men and women, respectively (Ferlay et al., 2010).

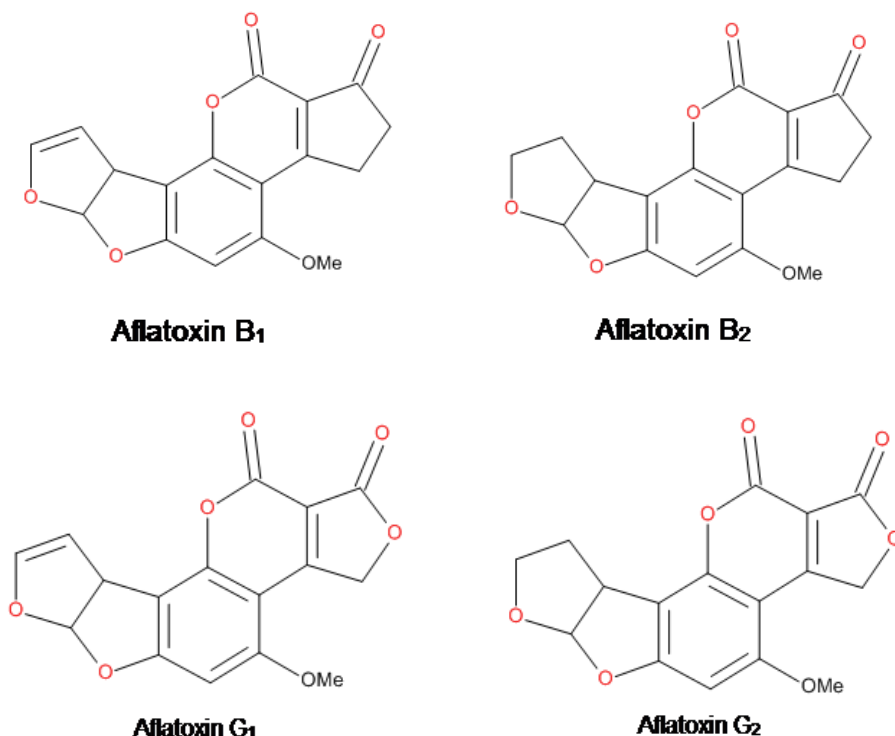
This paper describes the toxicological profile of AFs and focuses on AFB₁, the most reactive. The first section is “Characteristics”, where the chemistry and molecular structure of AF compounds are described. “Sources” identifies the *Aspergillus* species that produce AFs and briefly considers AF biosynthesis; “Occurrence” describes the environmental conditions that favor fungal growth in crops and lists commonly contaminated foods. Sections on “Environmental Fate” and “Exposure” follow. “Distribution and Elimination” discusses the metabolic pathways to non-toxic metabolites and excretion from the body. “Target Site and Mechanism of Action” addresses the conversion of

AF to the reactive AF epoxide and concomitant toxicity to the liver. To conclude, AF regulations in the USA and abroad are reviewed.

Characteristics

The predominant AFs that contaminate food and feeds are AFB1, AFB2, AFG1, and AFG2 (**Figure 2.1**). These heterocyclic compounds include a lactone moiety and are highly oxygenated, and AFB2 and AFG2 are dihydro-derivatives of AFB1 and AFG1, respectively. AFB1 and AFB2 have a cyclopentone ring on the lactone ring of the coumarin structure, whereas AFG1 and AFG2 have a second lactone ring in that position (Campbell & Hayes, 1976). The planar structure of all four compounds yields fluorescence under a black light: AFB1 and AFB2 fluoresce blue, and AFG1 and AFG2 fluoresce yellow-green (Nicolás-Vázquez, Méndez-Albores, Moreno-Martínez, Miranda, & Castro, 2010). AFB1 is the most toxic of the four forms followed by AFG1, AFB2, and AFG2, in decreasing toxicity (Dickens & Jones, 1963). AFB1 has a molecular weight of 312.28 g/mol and molecular formula $C_{17}H_{12}O_6$.

Figure 2.1: Aflatoxins B₁, B₂, G₁, and G₂



Sources

Aflatoxigenic species of *Aspergillus* include *A. flavus*, *A. parasiticus*, *A. nomius*, and *A. tamarii* (Payne & Brown, 1998), though *A. flavus* and *A. parasiticus* are the most studied. Genetic and biosynthetic pathways of AF production are well reviewed (Abrar et al., 2013; Bhatnagar, Ehrlich, & Cleveland, 2003; Payne & Brown, 1998). Briefly, AF biosynthesis involves genes in a 70 kb-region of the *Aspergillus* genome, encoding various protein classes such as dehydrogenases, polyketide synthases, monooxygenases, and fatty acid synthases. Complete biosynthesis of AF requires 21 enzymatic steps (Bhatnagar et al., 2003), and requires the transcription factor *aflR* (Ehrlich, Cary, & Montalbano, 1999). Temperature is the most critical environmental parameter known to influence AF biosynthesis (Schmidt-Heydt, Rüfer, Abdel-

Hadi, Magan, & Geisen, 2010). Increased expression of AF biosynthetic genes occurs between 28 and 30° C, and consequently, in that range toxin production is optimal (O'Brian et al., 2007). Though fungal growth increases as temperature approaches 37° C, *A. flavus* and *A. parasiticus* lose the capacity to produce their toxin at 37° C (Schmidt-Heydt et al., 2010), above which *aflR* becomes nonfunctional (O'Brian et al., 2007) .

Occurrence

A. flavus and *A. parasiticus* infection and toxin production occur in crops before and after harvest, and contamination has been reported in many agricultural commodities and animal feeds (Wood, 1992). Those principally include peanuts and maize, and to a lesser extent, rice, sorghum, and spices (Kumar, Basu, & Rajendran, 2008). AFs can occur in animal feeds derived from grains, oil seeds and their press cakes (Rodricks & Stoloff, 1977). Among lactating animals consuming such feeds, AF metabolites, including the hydroxylated metabolites AFM1, are found in dairy products (Fink-Gremmels, 2008). Animals excrete AFM1 up to four days following exposure, and between 0.3% and 2.2% of AF ingested by a dairy cow enters its milk (Yiannikouris & Jouany, 2002). AFM1 is designated as a possible carcinogen to humans by the International Agency for Research on Cancer (International Agency for Research on Cancer, 2010). Contamination also occurs in medicinal plants, tea, garlic, ginseng, ginger powder, and chili peppers in the *Capsicum* genus (Trucksess & Scott, 2008).

AF occurrence can be bifurcated into two phases: 1) initial plant development (pre-harvest) and 2) crop maturation shortly before and after harvest (referred

to in the present text as post-harvest occurrence) (Cotty & Jaime-Garcia, 2007). Diener *et al.* reviewed pre-harvest epidemiology of toxigenic *Aspergillus* infection and AF contamination, discussing important factors such as fungal substrates, field temperature, harvest timing, moisture, and insect damage (Diener et al., 1987). The literature on pre-harvest epidemiology is briefly mentioned here. Hot and dry environments are predictive of pre-harvest AF, which occurs even in apparently undamaged kernels (Cole, Sanders, Hill, & Blankenship, 1985), because temperature requirements of AF biosynthesis are met and water stress weakens defensive plant structures. For example, maize cultivated under drought conditions is more likely to have lateral splits in the kernel pericarp, exposing the inner kernel to fungal infection and arthropod infestation (Odvody, Spencer, & Remmers, 1997). Hill *et al.* found that neither drought stress nor hot temperatures alone resulted in more prevalent AF contamination in sound, mature peanut kernels; however, elevated heat in combination with dry soils resulted in highly prevalent contamination, even in edible-grade kernels (Hill, Blankenship, Cole, & Sanders, 1983). Furthermore, insect damage to crops exacerbates exposure to pathogenic fungi (Lillehoj & Hesseltine, 1977).

During the post-harvest stage, humid conditions favor infection and contamination (Cotty & Jaime-Garcia, 2007). Adequate drying and storage of agricultural commodities and sorting of kernels with visible mold are necessary to limit post-harvest AF contamination (Dickens 1977). Among subsistence farmers, especially in resource-limited countries, storage structures and practices influence post-harvest AF contamination (Hell, Cardwell, & Setamou, 2000; Udoh, Cardwell, & Ikotun, 2000).

Environmental Fate

AF in soil rapidly degrades to less toxic metabolites and cannot be extracted from soil four days after addition and incubation (Angle & Wagner, 1980). Soil type and microbial activity affect AF biodegradation: mineralization to carbon dioxide is greatest in silt loam soils, followed by sandy loam soil and silty clay loam (Angle, 1986). Microbial community composition in soil influences the rate of biodegradation and Doyle *et al.* have reviewed microorganisms capable of metabolizing AF to less toxic forms (Doyle, Applebaum, Brackett, & Marth, 1982).

Exposure

Among adults, common sources of exposure include contaminated peanuts and maize (Williams et al., 2004). Among neonates and very young children, dietary sources of exposures are AFM1 in human milk and dairy products, and maize in weaning foods (Gong et al., 2003). *In utero* exposure is supported by evidence of aflatoxin biomarkers in umbilical cord blood and placenta (Wild et al., 1991). Less commonly, respiratory exposure to AF occurs by inhalation of AF adsorbed to particles (Baxter, Wey, & Burg, 1981). The risk of this route of exposure is greater among agricultural workers in contact with contaminated corn silage and oil seed cakes (Kussak, Andersson, & K., 1995; Lanier et al., 2010).

Because AFs are not evenly distributed in stored food, analysis of food samples alone is a limited predictor of AF exposure. Validated AF biomarkers excreted in urine, such as AFB1-DNA adducts and AFM1, or circulating in

blood, such as AF covalently bound to blood albumin, have become essential indices of human exposure. Analyses of AF biomarkers are discussed at length in Chapters 6, 7 and 8. The reader is referred to a few extensive reviews (John D Groopman, Kensler, & Wild, 2008; Thomas W Kensler, Roebuck, Wogan, & Groopman, 2011; Paul C Turner, Flannery, Isitt, Ali, & Pestka, 2012; Wild & Turner, 2002). Indeed, biomarkers are valuable in examining consumption of contaminated foods and demonstrating epidemiological correlation between exposure and adverse health effects, both acute and chronic.

Health effects of acute and chronic exposure are well-documented among populations of endemic AF exposure in Africa and Asia (Williams et al., 2004). Acute exposure causes abdominal discomfort, anorexia, general malaise, low-grade fever, jaundice, and dark urine (Ngindu et al., 1982). Extremely high exposure, demonstrated by elevated AF-albumin circulating in blood, causes acute hepatic failure and death (Azziz-Baumgartner et al., 2005). Chronic exposure increases odds of hepatocellular carcinoma (HCC) (J D Groopman, Donahue, Zhu, Chen, & Wogan, 1985; Qian et al., 1994; Ross et al., 1992). In regions where chronic exposure is endemic, blood AF-albumin is associated with parameters of immune dysfunction (Jiang et al., 2005; P. C. Turner, Moore, Hall, Prentice, & Wild, 2003) and growth suppression, such as low birth weight, stunting, and wasting (Gong et al., 2003; Paul C. Turner et al., 2007). A contemporary research topic is the role that AF exposure plays, likely in conjunction with exposure to other mycotoxins, in environmental enteropathy, or insult to the absorptive capacity of the gut (Smith, Stoltzfus, & Prendergast, 2012).

Distribution and Elimination

Metabolism and distribution of AF are well-described by Dutton and McLean, but a brief overview is presented here (McLean & Dutton, 1995). In rats, ingested aflatoxin is absorbed in the intestine and most efficiently eliminated from the lower intestine via the duodenum (Kumagai, 1989), after which AF is absorbed by the liver. Experimental rat models have shown excretion rates of AFs at 54% and 15% via feces and urine, respectively (Coulombe & Sharma, 1985).

AFB1 undergoes phase I metabolism in the liver and is converted to activated *exo*-8,9-epoxide (see “Target Site and Mechanism of Action”), or to less-toxic metabolites that are generally more polar, by mixed function monooxygenases such as cytochrome P450 (CYP). Structurally, CYP proteins have approximately 500 amino acid residues, a heme group bound through the thiol of a cysteine residue at the C-terminus, and a hydrophobic domain on the N-terminus that binds to membranes in the endoplasmic reticulum (Hasler, J.A., Estabrook, R., Murray, M., *et al.*, 1999). Functionally, CYPs are monooxygenases with roles in metabolism of xenobiotics, drugs, steroid compounds, and lipids, among other substrates, and mediate oxidation via recruitment of electrons from NADPH and NADH to cleave molecular oxygen. Following CYP oxidation of substrate, water is formed (Hasler, J.A., Estabrook, R., Murray, M., *et al.*, 1999).

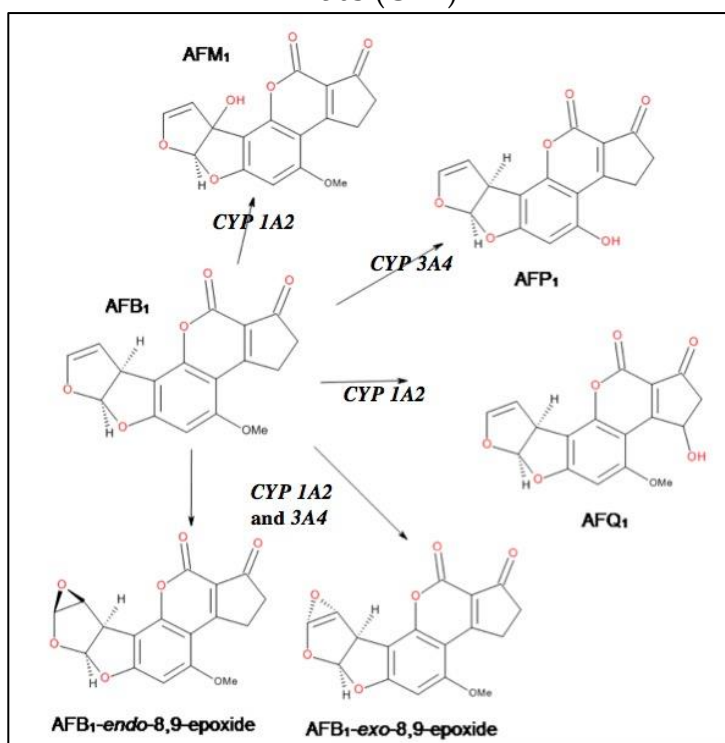
An extensive review of specific CYP isoforms and their substrates, including AFB1, is offered by Guengerich (Guengerich, 2005). CYPs 1-4 are generally responsible for Phase I xenobiotic metabolism and are genetically

polymorphic. CYPs 5-51 are highly conserved and are involved in endogenous substrate metabolism (Ingelman-Sundberg, 2004). The less reactive AF metabolites produced by CYPs include AFP1 (by CYP 3A4), AFQ1 (CYP 1A2), and AFM1 (CYP 1A2) and AF *endo*-8,9-epoxide (CYP 1A2 and 3A4) (Guengerich et al., 1998; McLean & Dutton, 1995; Raney et al., 1992; Ueng, Shimada, Yamazaki, & Guengerich, 1995) (**Figures 2.2**). Within the CYP super-class, which includes at least 57 CYP families identified in humans (Guengerich, 2005), CYPs 1A2 (Ueng *et al.*, 1995), 2A6 (Ingelman-Sundberg, 2004), and 3A4 (Ueng *et al.*, 1995) are known to facilitate oxidation of AF. There is evidence that fetus-specific CYP 3A7 also activates AFB1 to its toxic epoxide (Li, Yokoi, Katsuki, *et al.*, 1997). But, because investigation of CYP activity to AF has been limited to the few aforementioned CYP isoforms, oxidation of AF by other CYPs cannot be ruled out (Rendic, 2002).

The *endo*-8,9-epoxide is forty to five hundred times less reactive with DNA than the *exo*-8,9-epoxide (Iyer et al., 1994; Johnson, Harris, & Guengerich, 1996). AF-epoxides that do not form DNA adducts undergo phase II metabolism, including conjugation to reduced glutathione (GHS) (Degen & Neumann, 1978); water addition, mediated by microsomal epoxide hydrolase (EPHX), forming AF-dihydrodiol (McGlynn et al., 1995), and glucuronidation, all of which expedite elimination of AF through urine and feces in both animal models and humans (J D Groopman et al., 1985; Mykkänen et al., 2005). The phase II pathway most critical to detoxification is AF-GHS conjugation. It is catalyzed by glutathione S-transferase (GST) and inversely proportional to AF-DNA adduct formation (T. W. Kensler et al., 1986; Raj, Clearfield, & Lotlikar, 1984). Passage of the AF-GSH conjugate from liver to kidney occurs

through the renal artery. Following hydrolysis of glutamate and glycine from the AF-GSH conjugate, cysteine conjugated to AF is acetylated to a mercapturic acid and is excreted in urine (Wang et al., 1999). Formation of the AF-albumin adduct, a critical biomarker of exposure that circulates in blood, begins with EPHX conversion of AF-epoxide to its dihydrodiol, whose terminal furan rings undergo slow hydrolytic ring opening to form AF-dialdehyde (Wild & Turner, 2002). AF-dialdehyde is the precursor to the AF-albumin adduct.

Figure 2.2: Products of AFB₁ phase I metabolism, mediated by cytochrome P450s (CYP)



Target Site and Mechanism of Action

At the liver, morphological changes following acute AF exposure include cirrhosis, necrosis, fatty infiltration, and bile duct proliferation (Cullen &

Newberne, 1994; Ilic, Crawford, Egner, & Sell, 2010; Whittaker, Marais, & Zhu, 2010). Molecular and biochemical insult occur, given extensive covalent binding of AF to guanine in DNA and RNA, as well as to hepatocyte proteins (Appleton, Goetchius, & Campbell, 1982). Acute, oral, lethal dose to 50% of experimental animals (LD₅₀) ranges from 0.3 to 9 mg/ kg body weight for rats (McKean et al., 2006). For ducklings, hamsters, and rabbits, the LD₅₀s are 0.37 mg/kg, 10.2 mg/kg, and 0.5 mg AFB1/ kg body weight (Wogan, 1966).

Chronic exposure to AF induces HCC, and the mechanism of action has been extensively studied *in vitro* and is supported by epidemiological measurements of biomarkers resulting from AF-induced mutation and hepato-genetic changes. AF effects liver toxicity following conversion of AFB1 to its *exo*-8,9-epoxide by CYP 3A4 and 1A2 (Ueng et al., 1995). Within the nuclear envelope the *exo*-8,9-epoxide of AFB1 intercalates DNA (Guengerich & Johnson, 1999; Guengerich et al., 1998), and upon proper orientation towards nitrogen at position 7 of guanine nucleotides, bimolecular nucleophilic substitution forms a covalent linkage (Iyer et al., 1994). At DNA sites where that mutagenic event occurs, the AF-N7-guanine adduct can undergo hydrolysis, leaving an apurinic site, and releasing 2,3-dihydro-2-(N7-guanyl)-3-hydroxyafatoxin B1 (AF-Gua) (Essigmann et al., 1977). AF-Gua is excreted in urine and represents an effective biomarker of exposure and cancer risk (J D Groopman et al., 1985). Alternatively, the adduct may undergo hydrolysis at the imidazole ring of guanine, forming AFB1-formamidopyrimidine (AF-FAPY), a more recalcitrant lesion (Alekseyev, Hamm, & Essigmann, 2004). Both AF-N7-guanine and AF-FAPY can be repaired by the nucleotide excision repair pathway, which is considered in closer detail by Bedard and Massey

(Bedard & Massey, 2006). Guanine to thymine transversion is the most common mutation following depurination where AF-N7-Gua adducts occur (Foster, Eisenstadt, & Miller, 1983) because adenine residues are preferentially placed opposite apurinic sites (Kunkel, 1984).

Development of HCC takes place over thirty years or more following carcinogenic molecular lesions; carcinogenesis is accompanied by a host of chronically accruing genetic lesions that cumulatively decouple regulation of the cell cycle (Thorgeirsson & Grisham, 2002). Evidence has emerged of AF-induced genetic changes that presage carcinogenesis. The clearest example is the mutational hotspot in codon 249 of exon 7 in tumor suppressor gene (*p53*), where an AGG to AGT transversion takes place (Puisieux, Lim, Groopman, & Ozturk, 1991). This transversion results in serine replacement by arginine in that location of *p53* protein and underscores a predominant genetic change incident in AF-induced liver neoplasms. Epidemiological data, using genetic analysis of human liver specimens (Ozturk, 1991) or cell-free DNA originating from lysed tumor cells (Kirk et al., 2005), show that *p53* Ser²⁴⁹ is almost completely absent among HCC cases in Europe and the USA, where AF exposure is very low (Montesano, Hainaut, & Wild, 1997), but very prevalent in areas of endemic AF exposure (Bressac, Kew, Wands, & Ozturk, 1991). Among HBV-positive individuals, those in regions with elevated AF exposure have a higher prevalence of *p53* Ser²⁴⁹ than those in areas of lower exposure (Ozturk, 1991). Among carriers of HBV, it likely plays a multiplicative role in AF-induced *p53* Ser²⁴⁹ incidence, though the mechanistic relationship between AF and HBV remains unresolved (Montesano et al., 1997). Additional mutations in oncogenes, such as *ras*, are also observed in HCCs (Shen & Ong,

1996).

Regulation

AFs are regulated in roughly 100 countries, and the *Codex Alimentarius* of the Food and Agriculture Organization of the United Nations (FAO) includes a comprehensive reference of national regulations on AFs in food and animal feed (Food and Agriculture Organization of the United Nations, 2010b). Some national regulatory agencies mandate limits exclusively on AFB₁, while others limit total AFs. FAO *Codex* guidelines for AF include: unprocessed nuts, such as peanuts, almonds, Brazil nuts, hazelnuts, and pistachios (15 µg total AFs/kg); “ready to eat” nuts (10 µg total AFs/kg); and milk (0.05 µg AFM₁/kg) (Food and Agriculture Organization of the United Nations, 2010a). US regulatory standards established by the Food and Drug Administration are shown in **Table 2.1**. Thirty-nine European countries regulate mycotoxins (FAO 2010). AF standards within the European Union (EU) are harmonized (Van Egmond, Schothorst, & Jonker, 2007) and dictate maximum allowable levels of AFB₁, AFM₁ and total AFs under the Common Regulation No. 1881/2006. Some of the EU regulatory standards are shown in **Table 2.2**.

Table 2.1: AF action levels in the United States for food and feed

Commodity	Total AF Concentration ($\mu\text{g}/\text{kg}$)^a
Milk (AFM1)	0.5
Foods	20
Peanuts, peanut products, pistachio nuts, brazil nuts	20
Corn, peanut products, and other feed ingredients for immature animals and dairy animals	20
Corn and peanut products for breeding beef cattle, swine and mature poultry	100
Corn and peanut products for finishing swine, weighing 100 lbs or more	200
Corn and peanut products for finishing beef cattle	300
Cottonseed meal for beef cattle, swine, and poultry regardless of age or breeding.	300

^a Source: USDA (2009).

Table 2.2: European Union maximum allowable AF levels for food

Commodity	AF Concentration ($\mu\text{g}/\text{kg}$)^a
Infant formula (AFM1)	0.025
Dairy products for non-infants (AFM1)	0.050
Processed baby foods (AFB1)	0.10
Peanuts and other oilseeds for direct human consumption (Total AFs)	4.0
Maize to be subjected to sorting or physical treatment before human consumption (Total AFs)	10.0

^a Source: European Union Commission Regulation (2006)

National regulatory agencies set food safety standards not only based on toxicology of contaminants but other considerations as well, such as economic ramifications of lowering contaminant limits, availability of testing facilities, and availability of food meeting proposed standards, among others (Van Egmond et al., 2007). For example, Wu predicted that the United States,

Argentina, and China would bear annual losses of \$120, \$75, and \$215 million, respectively, if they were required to adopt the European Union AF standards for peanuts alone (Wu, 2004). Nations with less stringent AF limits, for instance many in Africa, bear direct economic costs of international regulatory differences, due to lost trade (Otsuki, Wilson, & Sewadeh, 2001). Nonetheless, for AF-regulated agricultural commodities, such as maize, international trade networks exist among nations with concordant regulations (Wu & Guclu, 2012).

Summary

AF is a mycotoxin produced by *Aspergillus flavus* and *Aspergillus parasiticus* and is a common contaminant of corn, peanuts, and cottonseed. Contamination occurs before harvest or during crop storage when temperatures are between 24° and 35° C. The principle route of exposure is via consumption of contaminated foods. Symptoms of acute, toxic exposure include abdominal discomfort, anorexia, general malaise, jaundice, acute hepatic failure, and death. Chronic exposure is a risk factor of hepatocellular carcinoma, and is also associated with growth suppression and immune dysfunction. Following absorption in the body, AF is either detoxified or activated by cytochrome P450 1A2 and 3A4. Detoxified metabolites are glucuronidated or conjugated with glutathione and excreted. The activated metabolite is the AF *exo*-8,9-epoxide. Mutagenesis occurs in hepatocytes via adduct formation with N7 of guanine. Acute and chronic liver damage include liver cirrhosis, lipid accumulation, bile duct proliferation and carcinogenesis. AFs are regulated in over 100 countries. Depending on the country and

commodity, maximum AF regulatory limits range between 0.025 and 300 $\mu\text{g}/\text{kg}$.

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CHAPTER 3: HAITI'S FOOD AND DRINKING WATER: A REVIEW OF TOXICOLOGICAL HEALTH RISKS¹

Abstract

Context. The Republic of Haiti is a developing country in the Caribbean region with a history that challenges toxicologists, yet the historical panoply of toxicological hazards in Haiti has received little scholarly attention. *Objectives.* The primary objectives of this paper are to review what is known about Haiti's current toxicological hazards, with a focus on chronic food-borne aflatoxin exposure and heavy metal contamination of water resources, and to compare these with previous large-scale, acute exposures to toxic substances: the 1995-1996 diethylene glycol intoxications (DEG) and the 2000-2001 ackee fruit poisonings. *Methods.* MEDLINE/PUBMED and the library website of Cornell University were searched using the terms "Haiti" and either "heavy metals," "aflatoxin", "diethylene glycol", or "ackee". The search was inclusive of articles from 1950 to 2012, and 15 out of the 37 returned were peer-reviewed articles offering original data or comprehensive discussion. One peer-reviewed article in press, 2 newspaper articles, 2 personal communications, and 1 book chapter from the personal databases of the authors were also referenced, making a total of 21 citations. *Results.* Elevated concentrations of aflatoxins (greater than 20 µg/kg) were documented for staples of the Haitian food supply, most notably peanut butters and maize. Human exposure to aflatoxin was confirmed with analysis of aflatoxin blood biomarkers. The

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implications of aflatoxin exposure were reviewed in the light of Haiti's age-adjusted liver cancer risk – the highest in the Caribbean region. Measurement of heavy metals in Port-au-Prince ground water showed contamination of lead and chromium in excess of the US Environmental Protection Agency's 15 µg/L Action Level for lead and 100 µg/L Maximum Contamination Level Goal for total chromium. The DEG contamination of paracetamol (acetaminophen) containing products in 1995-1996 claimed the lives of 109 children and the 2000-2001 epidemic of ackee fruit poisoning resulted in 60 cases of intoxication. *Lessons for the Haitian Government.* The DEG and ackee epidemics overwhelmed local Haitian public health resources. Yet, periods of eight and four months, respectively, passed before the Haitian government sought assistance following the initial poisonings. To our knowledge, the Haitian government did not enact policy to promote drug safety and prevent future poisonings. This likely will not change in the near future because of the state's finance and personnel crises. While protection of its people remains the prerogative of the Haitian government, it is extremely limited in managing chemical exposure to environmental toxins, including aflatoxin and heavy metals. *Conclusions:* The cases of DEG and ackee fruit poisoning demonstrate that environmental exposures to chemicals have occurred in Haiti. Current low-level exposures to aflatoxin and heavy metals highlight the risk that large-scale poisonings can occur. While awareness of toxicological hazards in Haiti must be acknowledged more widely within the government and non-governmental sectors, the lessons of these exposures are relevant to all developing countries where the capacity to discern and manage toxicological risks is absent or not yet effective.

Context

The island nation of Haiti is located on the Caribbean island of Hispaniola, shared with the Spanish-speaking Dominican Republic to the east. A French slave colony from 1697 to 1804, Haiti became the second republic in the Americas but today is the poorest country in the Western Hemisphere. With a population of 9.8 million, the 2011 gross domestic product was \$12.58 billion; the neighboring Dominican Republic, with a population of 10 million, had a gross domestic product of \$94.58 billion. The 7.0 magnitude earthquake of January 12, 2010 resulted in \$7.8 billion in damage and 5.4% contraction of the economy (Central Intelligence Agency, 2013). The public health sector is charged with the onerous burden of managing the response to an array of infectious diseases (Agarwal, McMorro, & Arguin, 2012; Ivers & Walton, 2012; Ocheretina et al., 2012), including cholera, malaria, and tuberculosis. Acute public health risks, such as infectious diseases, strain Haiti's resources, yet chemical exposures, too, pose a threat to public health that risk managers and aid organizations in Haiti must eventually address.

Haiti offers cases of interest to toxicologists, with particular relevance to nutritional toxicology, environmental chemistry, regulatory toxicology, rural development, and international health. These exposures to natural toxins and synthetic chemicals continue to put Haitians at risk, and several examples shed light on the realities of risk management with respect to toxic chemicals in developing countries. Haiti first elicited the interest of modern environmental toxicologists in 1963, when Stein *et al.* (Stein, Miller, & Fetzer, 1966) assessed blood cholinesterase activities of Haitian workers of the

National Malaria Eradication Program, which included the application of the organophosphate insecticide, dichlorvos. Tetrodotoxin, mercury, and cadmium have been detected in powders used for ceremonies of voodoo, a popular spiritual tradition among Haitians (Benedek & Rivier, 1989; Tarabar & Su, 2003). Methanol contamination of the local liquor clarin has been recorded, and the Pan American Health Organization estimated that 20 to 30 people died between January and March of 2011 due to methanol poisoning (World Health Organization/Pan American Health Organization, 2011). Formerly thought to be rare or anomalous, these and the lengthening list of recent cases provide increasing evidence for the systemic risk of environmental chemical exposures that Haitians bear. Furthermore, our concern is that the cases of poisonings in Haiti are not viewed within the context of one another; identification of patterns among all documented poisonings in Haiti is essential.

Objectives

The primary objective of this paper is to review what is known about Haiti's current non-microbial toxicological hazards and their concomitant public health risks. We focus on two current chemical hazards for which data exist: aflatoxin exposure and heavy metal contamination of water resources. The second objective is to compare these incidents with previous large-scale exposures to toxic substances that are well documented by toxicologists: the 1995-1996 diethylene glycol (DEG) poisonings and the 2000-2001 ackee fruit episode. Our final objective is to interpret lessons from previous poisonings to inform future interventions for current toxicological risks in Haiti.

Methods

The database MEDLINE/PUBMED and the library website of Cornell University were searched using the keywords "Haiti" and either "heavy metals," "aflatoxin", "diethylene glycol", or "ackee". Our search returned 37 peer-reviewed articles, 15 of which included original data or offered lessons learned from the DEG or ackee poisonings. Articles that briefly mentioned 1 of the 4 poisoning events or focused on wildlife exposure were not selected for review. The authors' personal collections provided 2 newspaper articles, 2 personal communications (not cited under References), 1 book chapter, and 1 peer-reviewed article in press. We therefore selected a total of 21 publications and personal communications that explicitly mentioned the Haitian cases of aflatoxin, heavy metal, DEG and ackee exposure. To estimate aflatoxin exposure, we used 3 databanks from the World Health Organization, the UN Food and Agriculture Organization, and the US Foreign Agriculture Service. In our results, we also included 13 references that did not cite 1 of the 4 Haitian cases but referred to US food and environmental quality standards; non-Haitian epidemiology of aflatoxin, heavy metals, ackee, and DEG; international pharmacovigilance; and Haiti's government, public health programs, and health statistics.

Aflatoxin

The risk of aflatoxin exposure in Haiti is high for several reasons. The climatic and agronomic conditions characteristic of *Aspergillus* infection and aflatoxin contamination are common in peanut producing regions of Haiti. These permissive conditions include: limited irrigation, stress caused by foliar

damage from other fungal infections, and warm, humid post harvest conditions. These have contributed to elevated concentrations of aflatoxin in Haitian peanut-based products and maize. Over four months during 1983 and 1984, Castor *et al.* (Castor, Mirocha, & Chang, 1987) collected 268 dry maize samples from 14 markets throughout Haiti and measured aflatoxin with HPLC. The authors found that 22% of samples had greater than 20 µg/kg aflatoxin and 10% contained greater than 100 µg/kg. The highest concentration of detected aflatoxin was 4,501 µg/kg, and the average was 124.1 µg/kg. The authors only reported the average incidence of aflatoxin B1 (88%) for all samples collected in the town of Gonaives. Filbert and Brown (Filbert & Brown, 2012) sampled Haitian peanut butters in 2010, and HPLC analysis confirmed that 8 out of the 10 Haitian samples were contaminated with greater than 20 µg/kg aflatoxin, the Action Level stipulated by the US Food and Drug Administration (US Food and Drug Administration, 2011). The most contaminated sample had 799.8 µg/kg total aflatoxins, and the mean and median levels of total aflatoxins were 260.6 and 268 µg/kg, respectively. Of aflatoxin detected, an average of 87% was aflatoxin B1.

The Haitian diet includes crops frequently contaminated with aflatoxin, namely peanuts and maize. The Food and Agriculture Organization of the United Nations (FAO) estimates that total peanut (in-shell) and maize production in 2010 were 19,000 and 233,700 metric tons (mt), respectively (Food and Agriculture Organization of the United Nations, 2011). Based on Haiti's 2010 population, per capita productions of in-shell peanuts and maize were 2.0 kg and 24 kg, respectively; but an estimate of per capita consumption, has been more elusive. Maize consumption is difficult to

calculate because a proportion of production may be given to animals or lost in processing and storage. An estimate of peanut consumption is also an approximation because a portion of total peanut yield is lost as the shell, depending on the maturity of the peanuts at harvest. For mature peanuts, up to 30% of the mass of a harvest consists of shell, but for immature peanuts, 30 to 45% of the mass consists of shell (Rhoads J, personal communication). Assuming that 30% of production mass is lost as the shell, annual per capita peanut production should be approximately 1.4 kg, or 3.8 g of peanut kernels per person per day. This is comparable to 2006 estimate by the World Health Organization (WHO) Global Food Contamination Monitoring and Assessment Program (World Health Organization, 2006). This program classifies countries into Food Cluster Diets. Based on earlier FAO data, the cluster in which Haiti is classified has a reported consumption of 2.9 g in-shell and 2.1 g shelled peanuts per person per day.

The volume of Haiti's imported peanuts and maize from its chief partner in trade, the United States, is much less than national production. According to the US Foreign Agricultural Service, maize exports to Haiti in 2008, 2009, 2010, and 2011 were 1121, 68, 0, and 47 metric tons (mt), respectively. US raw peanut exports to Haiti were 0 mt from 2008 to 2011, 2.5 mt in 2007, and 2.9 mt in 2006. More peanut butter was exported to Haiti: 35.9 mt in 2011, 214.8 mt in 2010, 53.6 mt in 2009, and 98.2 mt in 2008 (US Foreign Agriculture Service, 2012). This suggests that Haitians mostly consume local maize and peanut products, which are not monitored for aflatoxin contamination.

A crude estimate of aflatoxin exposure in Haiti can be made based on the 2010 FAO production data and the findings of Castor *et al.* (Castor et al., 1987) and Filbert and Brown (Filbert & Brown, 2012). The total estimated amount of ingested aflatoxin B1 is 8.07 micrograms aflatoxin B1/day, or 115 ng aflatoxin B1/kg body weight/day for a 70 kg individual. This level of aflatoxin exposure is within the range of dietary aflatoxin levels estimated in other countries where aflatoxin exposure and the association with liver cancer has been explored (Van Rensburg et al., 1985). For example, van Rensburg *et al.* (Van Rensburg et al., 1985) reported a crude hepatocellular carcinoma rate of 17.7 cases per 10^5 /year in the Mozambican area of Homoine-Maxixe, where estimated aflatoxin B1 intake was 131.4 ng/kg body weight/day. Where intake was 3.5 ng/kg body weight/day, as in the Kenyan high altitude area, the hepatocellular carcinoma rate was 1.2 cases per 10^5 /year. Comparing aflatoxin ingestion and the liver cancer rate of several African countries, as reported by van Rensburg *et al.* (Van Rensburg et al., 1985) an estimated 115 ng aflatoxin B1/kg body weight/day places Haitians at considerable risk of hepatocellular carcinoma.

Government surveillance and regulation of aflatoxin contamination in Haiti of popular commercial foods is non-existent. The authors are not aware of any governmental actions to limit aflatoxin exposure to the Haitian populations, nor have any international agencies, such as the FAO, established programs to limit aflatoxin contamination (Pretto, MPN, personal communication). On the other hand, Haitian producers of peanut-based ready to use therapeutic food, such as the non-profit organizations Meds & Food for Kids and Partners in

Health, do control and test their products for aflatoxin contamination (Charles, 2012; Rice, 2010).

Aflatoxin biomarkers have also been detected in blood serum samples from Haiti. Schwartzbord *et al.* (Schwartzbord et al., 2014) sought to determine the prevalence of aflatoxin covalently-bound to circulating blood albumin (measured as aflatoxin-lysine adducts, an established marker that persists as part of an aflatoxin-albumin adduct for up to 2 months following exposure). A second objective of that study was to characterize the relationship between the marker and consumption of maize and peanuts. Blood samples were obtained from 178 patients at Les Centres GHESKIO in Port-au-Prince, and patients were asked to recall the frequency of their maize and peanut consumption and the number of days since consumption within 14 days of participation. Using a nominal logistic model, it was shown that the detection of the biomarker was dependent on the frequency of peanut consumption as recalled by the subjects. Analysis of the variance and regression of individuals with detectable biomarker levels showed a significant effect between the log of detectable aflatoxin-lysine and frequency of peanut consumption. No significant relationship was observed between maize consumption and biomarker level. A study is underway to measure aflatoxin biomarkers in urine samples obtained from GHESKIO patients.

The prevalence of liver cancer in Haiti necessitates that further research elucidate the etiological role that aflatoxin plays. In their analysis of the GLOBOCAN database, Phillips *et al.* (Phillips et al., 2007) showed that the age-adjusted liver cancer incidence rate in Haiti is the highest in the Caribbean.

The GLOBOCAN database included the 2002 age-adjusted rates for 26 cancers in 172 countries. The authors compared the rates of 8 cancers for the US and 8 Caribbean countries, including Haiti. Among Haitians, the age-standardized rates for liver cancer incidence was 27.9 per 100,000 males, and for liver cancer mortality 26.8 deaths per 100,000 males. Compared to rates of other cancers in the Caribbean, liver cancer in Haiti has the third highest incidence and second highest mortality rate for men.

A strong interaction has been observed between aflatoxin biomarker levels and hepatitis B virus positivity (Qian et al., 1994). Aflatoxin is believed to be a causative agent in 4.6-28.2% of the world liver cancer burden, but calculating the incremental risk of additional amounts of aflatoxin at the country level requires knowledge of hepatitis B virus prevalence, among other etiological factors of liver cancer (Liu & Wu, 2010). The Pan American Health Organization (PAHO) reported that 5.5% of individuals tested in 1990 were positive for the HBV surface antigen and that, in 1996, 2-7% of pregnant women tested by Les Centres GHESKIO and the Child Health Institute were positive for HBV surface antigen (Pan American Health Organization, 1998). But because these data are neither representative of the population nor recent, calculating the contribution of aflatoxin exposure to liver cancer incidence in Haiti remains a challenge.

Heavy metal contamination²

In Port-au-Prince, groundwater pollution by heavy metals is a well-known environmental issue and poses a substantial risk to local resource users (Emmanuel, Angerville, Joseph, & Perrodin, 2007; Emmanuel, Pierre, & Perrodin, 2009). Indeed, Port-au-Prince groundwater resources are exposed to polluted effluents such as leachates, cesspools and septic tanks, storm water runoff, waste oil discharging, over-irrigation and industrial discharging.

Emmanuel *et al.* (Emmanuel et al., 2007) collected water samples over 3 day periods, once during December 2003 and again during April 2004. Collection took place from a water tank that served a population of 90,000, as well as 5 domestic water taps in the same area. The water tank had a mean lead concentration of 245 µg/L, and two of the water taps had mean lead concentrations of 45 and 185 µg/L. Another study on the impact of urban contaminants on water quality showed that the lead concentration was 10 – 40 µg/L, the nickel concentration was 15 – 250 µg/L and the chromium concentration was 18 – 470 µg/L in the discharge from the well of a Port-au-Prince emergency hospital during a sampling campaign from 2002 to 2005 (Emmanuel et al., 2009). The well was supplied by a private drinking water supply network. Additionally, the authors sampled the septic tank system, which had lower concentrations of heavy metals: lead 10-15 µg/L, nickel 30-180 µg/L, and chromium 180-440 µg/L. From a regulatory perspective, Port-au-Prince waters therefore can exceed the US Environmental Protection

² Prof. Evens Emmanuel composed the section “Heavy metal contamination.” Prof. Dan Brown and JRS edited it.

Agency established Action Level for lead of 15 µg/L and a Maximum Contamination Level Goal for total chromium of 100 µg/L in drinking water (US Environmental Protection Agency, 2012).

Outside of Port-au-Prince, Eisen-Cuadra *et al.* (Eisen-Cuadra, 2013) measured 26.24 to 198.44 mg/kg chromium in a lake sediment core from Étang Saumatre. While this study is based on a very limited sample of lake sediment, it points to the paucity of baseline data on heavy metal contamination in Haiti. We found one peer-reviewed paper that confirmed lead exposure among Haitians (Choulot & Carbonnier, 2007). Choulot and Carbonnier tested 24 children for lead in their blood from 2005 to 2006 in France. The children were tested within one month of arrival from Port-au-Prince, and lead concentrations ranged from 102 and 236 µg/L among 9 of the children. The US Centers for Disease Control and Prevention (CDC) has established a reference level of 5 µg/dL (50 µg/L) to identify children whose blood levels are elevated compared to most children in the US population (US Centers for Disease Control and Prevention, 2012). The picture emerging from these studies informs the pressing need for continued health risk assessment of the urban water supply as well as monitoring of human exposure to heavy metals.

Paracetamol (acetaminophen) contamination with DEG

In Haiti, DEG contamination of two paracetamol syrups caused the deaths of up to 109 children, including 87 confirmed and 22 possible cases (O' Brien et al., 1998). From November of 1995 to May of 1996, 32 children were admitted to the University General Hospital in Port-au-Prince for acute renal failure,

signaling the first signs of the DEG poisoning epidemic. Symptoms included anuric renal failure, pancreatitis, hepatitis, and neurological dysfunction (Centers for Disease Control and Prevention, 1996). Some DEG poisoning victims were taken to the United States for care, and Scalzo (Scalzo, 1996) described the clinical presentation of these patients. The Haitian Ministry of Public Health (MSPP, for its French acronym), conducted a recall and public information campaign in June and July of 1996, relying on the assistance the Pan American Health Organization (PAHO), CDC, and US Food and Drug Administration (FDA). The unfolding of international support to the MSPP, which led to the rapid and effective recall and investigation of DEG poisoning, serves as one example in which epidemic assistance prevented further risk of chemical exposure to the Haitian people.

Based on interviews with CDC and FDA officials, Junod (Junod, 2000) described the events of the outbreak, including the development of CDC and FDA responses. The CDC involvement with the DEG intervention began during June 1996 upon invitation by the PAHO. The CDC directed an investigation and showed causal relationship between the deaths and two paracetamol-containing syrups, Afebril and Valodon. Both were manufactured in Haiti. Following consultation with the PAHO and CDC, the Haitian government issued a public alert against Afebril and Valodon on 22 June 1996. The CDC obtained from patients and pharmacies 200 samples of the two medicines and sent them to the National Center of Environmental Health of the CDC for chemical analysis. DEG concentrations in syrup samples ranged from 4% to 17%, and on 1 July 1996, analysis of the medicines' ingredients revealed that the source of contamination was glycerin

contaminated with 24% DEG (Barr et al., 2007). In tracing the origin of contamination, the FDA National Drug Expert concluded that the contaminated glycerine had been manufactured in China but arrived to Haiti after having been sold, purchased, and resold by multiple European chemical brokers (Scalzo, 1996).

In their comparisons of global DEG poisonings, both Schier *et al.* (Schier, Rubin, Miller, Barr, & McGeehin, 2009) and Wax (Wax, 1996) made the case that the pharmaceutical industries of developing countries, especially Haiti, are vulnerable to poor safety when lesser quality, inexpensive chemical ingredients are available. Wax implores us to learn from the past and consider a perennial challenge: developing nations have limited resources to monitor pharmaceutical safety. Over 15 years have passed since the DEG epidemic, but few have asked whether Haiti is any more capable in assuring the safety of its pharmaceuticals and, more broadly, its food.

There is no evidence that the Haitian government has improved regulatory capacity to prevent the sale of contaminated pharmaceuticals. In 2012 Hoffman *et al.* (Hoffmann, Fouretier, Vergne, & Bertram, 2012) reviewed pharmacovigilance regulations in 21 countries in Latin America and the Caribbean, including Haiti. For each country, the authors assessed if healthcare professionals and pharmaceutical manufacturers were legally required to report adverse drug effects to the government. Countries were ranked as having high, medium, or low level requirements. Haiti was one of five countries to rank low, having no reporting activities implemented. The findings of Hoffman *et al.* (Hoffmann et al., 2012) highlight that little progress

has been made to promote long-term drug safety in Haiti, and the regulatory deficiency of Haiti's drug safety continues despite international support to promote pharmacovigilance, such as the WHO Programme for International Drug Monitoring. The program focuses on educating and training member countries to establish national pharmacovigilance systems and includes 108 member countries and 34 associate member countries. Haiti is neither a member nor associate member of this program (World Health Organization, 2012).

Ackee fruit poisoning

The most widespread event of ackee poisoning in Haiti occurred from November 2000 to March 2001. An overview of the poisoning and two subsequent investigations were documented by the Pan American Health Organization (PAHO) (Moya, 2001). In February 2001, the Haitian Ministry of Public Health (MSPP) investigated the epidemic and registered 73 cases. A month later, the CDC, the PAHO, and the MSPP conducted a second investigation and used a stricter case definition (Joskow, Belson, Vesper, Backer, & Rubin, 2006). Joskow *et al.* detailed the case identification process, reviewed outbreak data by local health officials, and identified risk factors. Of the 105 potential cases, 60 met the case definition. The ages of these 60 individuals ranged from 16 months to 88 years old, and the mean and median ages of case-patients were 15 and 7 years old, respectively. Joskow *et al.* noted comments by local health officials, who stated that a 10-day period of heavy rains preceded the first poisoning cases by 2 weeks. The officials maintained that the heavy rains resulted in damage to crops and livestock, causing a local food shortage and consumption of unripe ackee fruit.

Lessons for the Haitian Government

In discussing Haiti's current food and environmental chemical hazards, the cross-case comparison of the DEG and ackee poisonings is instructive. In both cases, acute, catastrophic poisoning occurred prior to the mobilization of national and international resources for chemical risk management. The DEG and ackee epidemics overwhelmed local public health resources (Becker, 1997). Yet, periods of eight and four months, respectively, passed before the Haitian government sought assistance following the initial poisonings. These cases suggest that the government will not seek resources until a catastrophic chemical exposure occurs.

To our knowledge, the Haitian government did not enact policy to promote drug safety and prevent future poisonings following the cases of DEG and ackee. While the government demonstrated capacity to seek assistance from the PAHO, FDA, and CDC, state capacity to prevent toxin contamination and exposure is very limited (Hoffmann et al., 2012). This likely will not change in the near future because of the state's sobering situation concerning finance and personnel: the UN Office for the Special Envoy to Haiti reported that foreign aid was 1.1 and 1.3 times the Haitian government's revenue for 2005 and 2009, respectively, and the governmental workforce decreased 33% due to fatalities and attrition within one year of the earthquake (UN Development Programme, 2010; UN Office of the Special Envoy for Haiti, 2011). While protection of its people remains the prerogative of the Haitian government, it

is extremely limited in managing chemical exposure to environmental toxins and toxicants, including aflatoxin and heavy metals. Lest disaster management perpetually remain the norm, the government will need to implement its own agencies to monitor environmental quality and food safety.

Conclusions

Awareness of toxicological hazards in Haiti must be acknowledged more widely within the government and non-governmental sectors. The cases of DEG and ackee fruit poisoning demonstrate that environmental exposures to chemicals have occurred in Haiti. Current low-level exposures to aflatoxin and heavy metals highlight the risk that large-scale poisonings can occur. While the four cases discussed relate directly to Haiti, the lessons of DEG and ackee fruit poisoning are relevant to all developing countries where the capacity to discern and manage toxicological risks is absent or not yet effective.

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PART 2

CHAPTER 4: AF CONTROL AND LIVELIHOODS AMONG PEANUT FARMERS IN NORTH EAST HAITI: A FORMATIVE SURVEY¹

Abstract

Aflatoxin (AF) is a mycotoxin produced by toxigenic *Aspergillus* spp. and represents a threat to the safety of peanuts in Haiti. Attenuation of AF contamination throughout the peanut value chain requires pre- and post-harvest controls, such as managements of foliar pathogens, adequate irrigation, kernel sorting, and adequate storage. We partnered with Meds & Food for Kids (MFK), a Haitian producer of Ready-to-use food, and sought to: 1) characterize a livelihood profile of farmers in the North and North East Departments of Haiti, and 2) examine the association between MFK Agricultural Programing and farmers' knowledge of AFs, and the extent to which that knowledge was associated with improved peanut production practices. Our survey of 109 farmers showed that the majority practice subsistence agriculture and rely on a limited set of traditional manual tools. Only 5.7% of farmers reported access to fungicides to control foliar pathogens. No farmers had access to irrigation. Forty percent of farmers cited inaccessibility to credit as a constraint to production, and overall our profile is suggestive of a very resource-limited farming system. Twenty-three percent of participants were aware of AF, and awareness was significantly associated

¹ I conducted this study in collaboration with Lora Iannotti of Washington University in St. Louis and her former research assistant Colleen Smith. Smith designed part of the survey instrument used for this study and oversaw data collection. I was responsible for survey questions concerning aflatoxin, conducted data analysis, and drafted this manuscript. Accordingly, I have used the first person plural tense in this chapter to reflect that partnership.

with participation in peanut planting training (Pearson test statistic=18.371, p-value< 0.0001), knowledge of fungicides (Pearson test statistic=10.690, p-value=0.0011), knowing the Haitian MFK agronomist (Pearson test statistic=17.145, p-value<0.0001), and knowing the American MFK agronomist (Pearson test statistic=8.531, p-value=0.0035). Wider spread capacity building, including transfer of agricultural technology and education, will be essential to establishing AF management practices among Haiti's farmers.

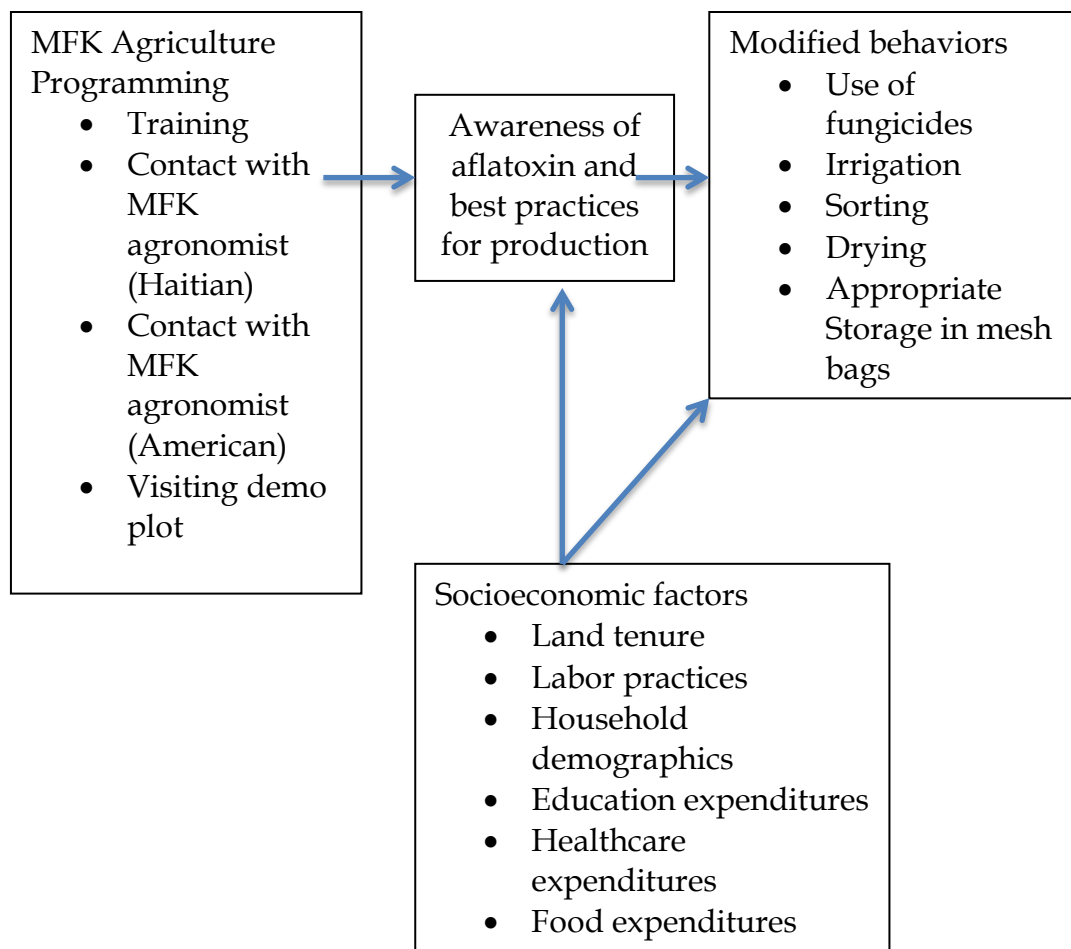
Introduction

Purpose

Aflatoxin (AF) is a carcinogenic mycotoxin that contaminates peanuts and maize in tropical areas (Sanders, Gorbet, Shokes, Williams, & McMeans, 1989), particularly where irrigation and pest management are absent prior to harvest and adequate food storage is limited (Hell, Cardwell, & Setamou, 2000). AF exposure has been implicated in numerous adverse health effects, including liver cancer, immune-dysfunction, and stunted growth (Wild & Turner, 2002). To maintain the safety of susceptible foods, abatement of AF contamination requires a coordination of best farming and storage practices throughout the peanut value chain. In the Caribbean island-nation of Haiti, farmers have produced peanuts for generations without access to irrigation or agricultural chemicals, and the predictive factors of post-harvest contamination are prevalent, including inadequate storage, loss due to pests, and rot (Pluviose, 1991). Those reasons are credited for low yields that peanut farmers experience and the alarming prevalence of AF contamination among Haitian peanut products (Filbert & Brown, 2012; Food and Agriculture Organization of the United Nations, 2011). Since 2003 Haiti's local procurer and producer of

Ready-to-use Foods (RUFs), Meds and Food for Kids (MFK), has coupled its purchase of peanuts in the North East Department of Haiti with agricultural programming to inform pre- and post-harvest practices of area farmers, with the dual purpose of improving farmers' yields and lowering the prevalence of peanuts highly contaminated with AF. We reviewed the results of a formative evaluation of farmer livelihoods and MFK Agricultural Programming (AP) that we conducted in partnership with MFK. The primary objective of the present report was to characterize a socioeconomic livelihood profile of peanut farmers in the North and North East Departments of Haiti. The secondary objective was to examine the association between MFK AP and farmers' knowledge of AFs, and the extent to which that knowledge was associated with improved peanut production practices. Our conceptual framework of MFK's agricultural programming model as a means to influence farmers' implementation of improved practices is summarized below (**Figure 4.1**).

Figure 4.1: Conceptual model of MFK agricultural programming and modification of pre- and post-harvest farming practices among Haitian growers.



A picture emerges in which stronger controls are needed to prevent AF throughout the entire peanut value chain in Haiti. For example, contamination in excess of the US Food and Drug Administration action level for AF has been documented for Haitian peanut products (Filbert & Brown, 2012) and maize (Castor, Mirocha, & Chang, 1987). Furthermore, analysis of blood AF biomarkers in Port-au-Prince confirmed Haitian exposure to AF and showed a strong association with the frequency of peanut consumption reported among study participants (Schwartzbord et al., 2014).

Prophylactic practices that limit AF contamination before and after harvest are well established. Those include pre-harvest strategies that encompass cultural controls to manipulate field environments and reduce *Aspergillus* infection of crops (such as adequate irrigation and drought resistance peanut varieties), chemical controls (such as fungicide application to treat foliar pathogens), and biological controls (such as application of atoxigenic *Aspergillus* to fields before planting)(Cole, Sanders, Hill, & Blankenship, 1985; Holbrook, Guo, Wilson, & Timper, 2009; Jacobi & Backman, 1994). Plant scientists have long sought to breed AF-resistant maize and peanut varieties. Such experimental efforts have been challenged by gene-environment interactions, whereby phenotypes conferring AF-resistance are compromised under field conditions (Nigam, Waliyar, Aruna, et al. 2009). Post-harvest strategies of AF contamination require cultural controls, such as timely drying of kernels following harvest, removal of contaminated kernels and adequate storage (Magan & Aldred, 2007). These simple practices have proven efficacious even for small-scale, resource-limited African farmers to decrease food contamination (Hell et al., 2000) and AF exposure (Turner et al., 2005).

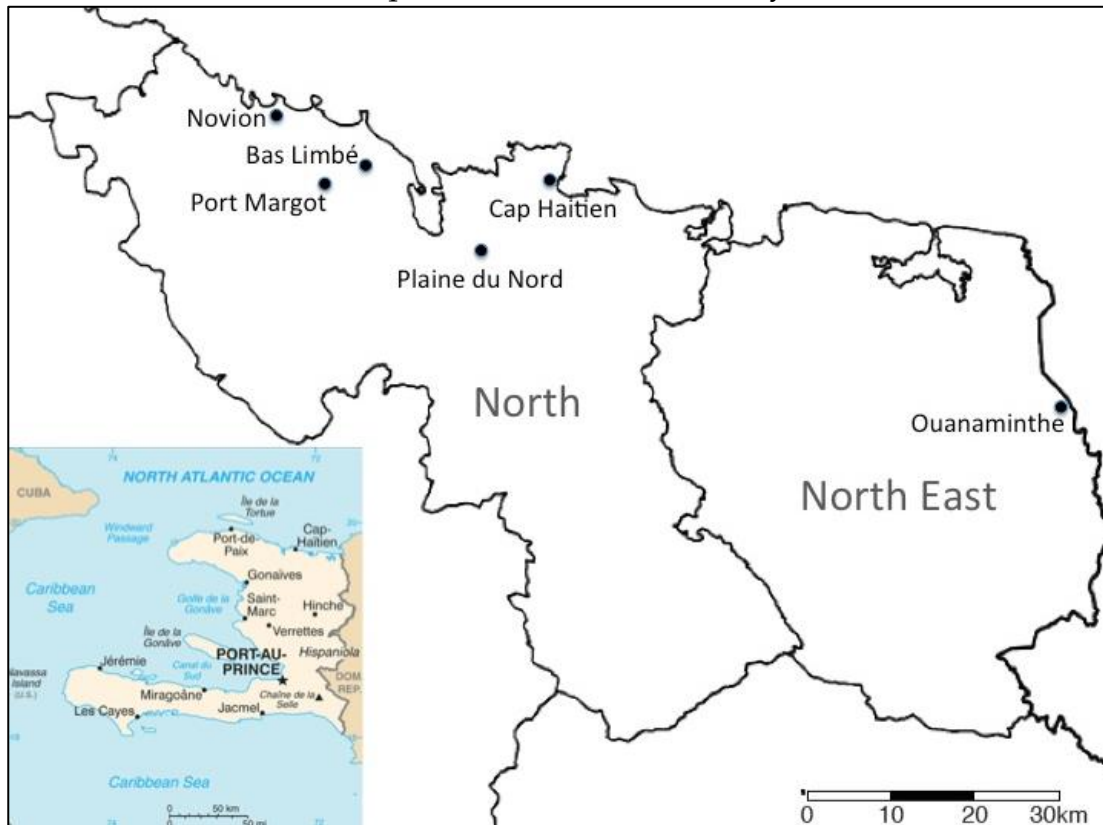
A few studies have shown associations between adoption of post-harvest practices and indicators of farmers' socioeconomic context. In Ghana, socioeconomic factors such as age, gender, and education were strongly predictive of peanut sorting to remove immature, damaged, or moldy kernels (Awuah, Fialor, Binns, Kagochi, & Jolly, 2009). Likewise, Kumar and Popat associated the adoption of AF management practices to socioeconomic and psychological factors among Indian farmers (G D S Kumar & Popat, 2010; G.D. Satish Kumar & Popat, 2010). Partnered with MFK in Haiti, we hypothesized that indicators of exposure to MFK AP and socioeconomic factors of farmers would be associated with awareness of AF.

Methodology

Study Area

Haiti is comprised of ten departments, and we conducted our study in the North and North East Departments, with sampling conducted around the towns of Bas-Limbe, Novion, Plaine du Nord, Port Margot, and Ouanaminthe (Capotille). These towns are located between latitudes 19° 32' and 19° 48' N and longitudes 71° 28' and 71° 42' W (see map in **Figure 4.2**). The North comprises an area of 2,105 km² and the North East, 1,623 km². Average rainfall ranges from 50.8 to 229 mm per month (World Bank, 2013a).

Figure 4.2: Map of villages in the North and North East Departments of Haiti where peanut farmers were surveyed.



To our knowledge, no peer-reviewed studies have documented Haitian agriculture in the North and North East Departments, though several general attributes of Haitian agriculture have been documented. Various development assistance organizations, such as the World Bank (World Bank, 2006, 2013a), International Monetary Fund (International Monetary Fund, 2008), and US Agency for International Development (US Agency for International Development, 2005), have produced demographic and agricultural profiles for Haiti. Average plot size is small, and the most recent survey of land tenure, conducted in 1995, found the average size of land to be 1.2 ha (Wiens & Sobrado, 1998). Farmers lack access to improved crop varieties, mechanized

farm equipment and synthetic pesticides and fertilizers (Raynolds, 1987; Waters, 1990). The ten crop categories of greatest national production are sugar cane; manioc and yams; bananas; sweet potatoes; plantains; maize; mangoes, mangosteens and guavas; rice; and fresh vegetables (Food and Agriculture Organization of the United Nations, 2010). While Haiti's national peanut production is relatively less than these,² peanuts contribute to diet quality in Haiti as a source of protein and fat, particularly among food insecure individuals unable to access animal-sourced foods. Peanuts are also the principle ingredient in lipid-based nutritional supplements that are standard treatment for malnourished children in Haiti (Iannotti, Dulience, Green, *et al.*, 2013). To our knowledge, farmer capacity among peanut producers in Haiti has been evaluated by only one other study, which considered the effect of peanut cooperative membership on farmer income in the areas of Capotille and D'Osmen, both in the North East Department (Pluviose, 1991). However, Pluviose made no mention of aflatoxin.

Survey Design

We chose a formative evaluation design (Rossi, Freeman, & Lipsey, 2004; Scriven, 1991) with the dual purpose of testing our hypotheses and obtaining information that would guide the expansion of MFK AP to a larger scale. The salient feature of MFK AP is its farmer-training program, which consists of demonstration plots and training modules. Trainings are led by Haitian and non-Haitian MFK agronomists and inform farmers of best practices in peanut production and post-harvest processing, including fungicide application, post-harvest drying and sorting.

² See Chapter 3 for FAO estimates of Haiti's annual peanut production.

A survey instrument was developed and administered in Haitian Creole. It included 113 questions and consisted of three sections: Livelihood, Peanut Production, and MFK AP Exposure (i.e., participation in farmer training). The Livelihood questions explored household size; number of children within a household and their age, sex, and education levels; household income and household expenditures; contribution of all household members to family income; and the growing of crops other than peanuts and the contribution of those different crops to family income. The Peanut Production section examined four key areas: 1) type of peanut varieties planted and mode of access; 2) production costs, including costs of labor and supplies; 3) yield per harvest and market access; 4) inputs and equipment for preparation, maintenance, and harvest of land. The MFK AP Exposure section assessed farmers' access to MFK agricultural extension activities, such as peanut demonstration plots and dissemination of best practices, to improve yield and better manage AF contamination. The survey was written in English and translated to Haitian Creole. Cognitive testing among a limited sample of individuals similar to that of the study population was performed to determine clear understanding of the questions being asked. Our study was submitted to the Cornell Institutional Review Board (IRB) and determined exempt from IRB review.

Sampling Design

The research team in partnership with MFK chose survey sites based on MFK's extensive networks among peanut farmers. In each zone where interviews were conducted, peanut farming was a traditional and widespread

practice, and there were existing networks of farmers who supplied MFK with local Haitian peanuts as part of the RUF supply chain. The number of farmers supplying peanuts to MFK, however, was known to vary among areas. One enumerator was selected to carry out the interviews and was accompanied by a local guide who served as an authority figure and respected leader in each community where the survey was conducted. The role of the guide was to assist in introducing the enumerator to an area where peanut farmers were working. Upon arrival at each site, the enumerator used a quasi snowball sampling method to select farmers for interviews, an approach that was chosen because a simple random sample of the area's peanut farmer population was not feasible. This approach served the purpose of our formative research design.

Interview Methods

A Haitian enumerator was trained to administer the survey, and our team reviewed each question with the enumerator. She was instructed to read questions and record responses on a paper copy and used a digital voice recorder for each interview. In our presence, the enumerator conducted practice surveys with farmers. Prior to interviews, the enumerator stated affiliation with Washington University in St. Louis, and wore a badge with her name. She did not state her affiliation with MFK. To avoid distractions to participating farmers, no member of the research team accompanied the enumerator during interviews.

Statistical Analysis

Data were entered in a Microsoft Excel spreadsheet, and descriptive statistics were tabulated (means, standard errors, and percentages) to summarize results from sections on Livelihood, Peanut Production, and MFK AP Exposure. Economic data were converted from Haitian Gourdes to US\$ using the 2011 currency exchange (41 Gourdes per US\$). Because income data were skewed, we applied natural log transformations (\ln) to achieve normality, and geometric means with 95% confidence intervals (CI) were calculated. For bivariate Chi-Squared analyses, Pearson values were calculated to test independence between participants' awareness of AF (i.e., "yes" or "no") and the following categories: participation in farmer trainings and visit of peanut demonstration plots (i.e., not restricted to MFK activities), knowledge of fungicides, knowledge of MFK agronomists, familiarity with MFK, gender, geographic location, education, and cooperative peanut marketing. To determine the dependence of income on AF awareness, income data and \ln income were regressed to the log odds of AF awareness. We did not expand our regression analysis to more complex regression models (i.e., socioeconomic indicators regressed to log odds of AF awareness) because our sampling method and design did not meet the assumption of independent observations. All statistical analyses were conducted using JMP 9 software (SAS 2010).

Results

Livelihood Profile of Participating Peanut Farmers

The participants from Bas-Limbe, Novion, Plaine du Nord, Port Margot, and Ouanaminthe totaled 24 (22% of total), 10 (9%), 27 (25%), 18 (17%), and 30

(28%), respectively. There were 20 (18%), 50 (46%), and 38 (35%) participants who had no schooling, primary education, and secondary education, respectively. One participant had received a university education. Of the 109 participating farmers, 29 (27%) were female and 80 (73%) were male. The age of participants ranged from 18 to 88 years old and the average was 45 (standard error=1.3). Summary statistics of household size are listed in **Table 4.1**.

For farmers who reported their weekly income (n=98), the geometric mean was US \$16 (95% CI: \$13 to \$20), equivalent to an annual income of US \$835. Sixty-two farmers (57%) practiced another vocation to supplement agricultural earnings while 47 (43%) did not. The most common jobs were merchant (19%), “other” (18%), and teacher (6%). Seventy-three farmers (80%) reported that members of their household contributed in paying expenditures. Summary statistics of household expenditures are presented in **Table 4.2** and suggest how participants prioritized major expenses, such as food, school costs for children, and health care.

We asked farmers (n=108) how much food was grown by the family for household consumption, and 12 (11%) responded “all of it,” 44 (41%) “most of it”, 51 (47%) “some of it,” and 1 (1%) “I do not own a garden.” Nearly all farmers (98%) reported production of crops besides peanuts. See **Table 4.3** for data on non-peanut crop production. Eighty-seven participants (80%) raised livestock and 47 (43%) raised cattle, 54 (50%) goat, 25 (23%) swine, 37 (34%) poultry, and 5 (5%) horses. Fifty-two farmers (48%) raised more than one

species of livestock. A summary of land tenure status is presented in **Table**

4.4.

Table 4.1: Summary statistics of household size of 109 peanut farmers surveyed in the North and North East Departments of Haiti.

Household Size	Mean (Standard Error)	Range
Number of family household members	6 (0.26)	0-15
Number of total household members	6.5 (0.27)	2-16
Number of household children ¹	3.7 (0.22)	0-15
Number of household family children	3.2 (0.20)	0-11

¹Includes children who are related and non-related to the head of household.

Table 4.2: Summary statistics of household income and expenditures among 109 peanut farmers surveyed in the North and North East Departments of Haiti.

Family Expenses	Mean (Standard error)	Median
Annual education expenditures per family (US\$) (n=84)	341(51)	198
Education expenditures per child per family (US\$) (n=84)	106(12)	70
Annual health care expenses (n=95)	118 (18)	73
Reported weekly food expenses (US\$)	28 (2)	24

Table 4.3: Number of harvests per year and revenue per harvest for crops besides peanuts among 109 peanut farmers sampled in the North and North East Departments of Haiti.

Crop	Number of Farmers	Mean Number of Harvests per Year (Standard Error)	Revenue per Harvest (Standard Error) in US\$
Manioc	34	1.2 (0.07)	74 (400, n=34)
Rice	32	1.2 (0.07)	2520 (500, n=23)
Sugar Cane	12	1.5 (0.19)	6283 (1898, n=12)
Corn	53	1.5 (0.07)	2106(371, n=43)
Banana	32	1.09 (0.07)	2252 (521, n=21)
Black Bean	57	1.4 (0.07)	2859 (961, n=42)

Table 4.4: Reported land tenure status among 109 peanut farmers surveyed in the North and North East Departments of Haiti.

Land Tenure Status	Number of Farmers (% of Total)
Own	31 (31%)
Rent	25 (23%)
Sharecrop	16 (15%)
Share family land	8 (7%)
Rent and share family land	1 (1%)
Sharecrop and share family land	1 (1%)
No response	21 (19%)

Peanut Production

Participants on average had been farming peanuts for 17 years (standard error=1.1). Farmers reported growing the Valencia-Spanish variety (18, or 17% of respondents), Runner variety (85, or 79%), or both varieties (4, or 4%). The Valencia-Spanish reaches maturity after 3 months, and the Runner after 5 months. There were 80 (74%) farmers who hired labor for planting, 99 (92%) for weeding, and 85 (79%) for harvest. Of farmers who used labor, 86 (88%) provided food. Most farmers (81, or 74%) exclusively sold peanuts at market, though 5 (5%) sold through a cooperative and 7 (6%) through a combination of cooperative and market.

Use of agricultural technology was limited among farmers to simple manual tools: 106 participants (97%) reported using a hoe, 31 (29%) a machete, and 14 (14%) a rake to prepare, maintain, or harvest their land. One farmer reported use of a traction animal. None of the participants had access to a tractor or irrigation. The tools ranked as most important for planting peanuts were the hoe (68% of farmers) and the machete (3.7%). Agricultural chemicals had been adopted by 16 farmers, and of those individuals, 13 cited pesticide use for insect control. Loss in peanut yield in northeast Haiti is largely due to foliar diseases, and subjects were questioned regarding their awareness of foliar fungicides, revealing that 5.7% of subjects were familiar with fungicides, 1.9% had experience using them, and 5.7% had access to them. Seventy-six farmers (70%) reported an interest in using fungicides to control foliar pathogens. Fifty-six farmers (51%) said they were aware that special protective clothing, such as rubber boots, mask, and protective jacket, would be required to safely

apply fungicides, while 52 (48%) were not (1 farmer abstained). No farmers reported owning protective clothing.

MFK AP Exposure

Twenty-two percent of farmers reported working with an organization to improve their peanut farming. Familiarity with our collaborating organization, MFK, was reported among 13 (12%) of farmers, and 58 farmers (54%) had heard of MFK's peanut-based RUF (*medika mamba*). Familiarity with MFK's Haitian and American agronomist was noted among 29 (27%) and 39 (36%) farmers, respectively. These two agronomists had maintained demonstration plots, of which 19 (18%) farmers reportedly knew. Farmers who reported attendance of peanut farming trainings numbered 28 (26%), and of these, 27 (96%) shared information from trainings with other farmers. Nearly all farmers (108, or 99%) expressed a desire to participate in future training.

Familiarity with AF in peanuts was found among 25 (23%) of participants. Because AF can be reduced in food by removing damaged and moldy peanuts, we asked if farmers sorted their peanuts. Nearly all farmers (108, or 99%) reported that they sorted peanuts. Ninety-one (83%) reported to throw away bad peanuts, and other uses included production of peanut butter, candy, grilled snacking peanuts, or other foods (15 respondents, or 14%). Use of bad peanuts for animal feed was also reported (3 respondents, or 2%). All respondents stated that family members consumed peanuts and 99 (94%) reported that their children consumed peanuts. As an open-ended question, farmers were asked what they needed to increase peanut production.

Responses included access to credit (40% of farmer responses); agro-chemicals (22%); equipment, tractors, or tools (19%); seed (7%); labor for planting or harvesting (7%) and training (6%). Some farmers mentioned multiple inputs needed, and 14% cited no inputs or technologies needed to improve production.

Association between AF Awareness and Indicators of Livelihood, Peanut Production and MFK AP Exposure

We rejected independence between AF awareness and the following variables: peanut planting training (Pearson test statistic=18.371, p-value< 0.0001), knowledge of fungicides (Pearson test statistic=10.690, p-value=0.0011), knowing the Haitian MFK agronomist (Pearson test statistic=17.145, p-value<0.0001), knowing the American MFK agronomist (Pearson test statistic=8.531, p-value=0.0035), and visiting a peanut demonstration plot (Pearson test statistic=15.163, p-value<0.0001). Chi-Squared analyses revealed significant and positive associations between training and knowing the Haitian MFK agronomist (Pearson=27.396, p-value<0.0001), knowing the American agronomist (Pearson=20.839, p-value<0.0001), visiting a demonstration plot (Pearson=12.270, p-value=0.0005), and working with an organization besides MFK (Pearson=5.699, p-value=0.0170). Selling peanuts through a coop was not associated with training among farmers (Pearson=0.006, p-value=0.9381).

We failed to reject independence between AF awareness and the following variables: gender (Pearson test statistic=1.193, p-value=0.2747), research town (Pearson test statistic=5.980, p-value=0.2006), farmer education (Pearson test

statistic=0.160, p-value=0.9231), knowing MFK (Pearson test statistic=2.057, P-value=0.1515), working with another organization (Pearson test statistic=1.201, p-value=0.2731), and selling peanuts through a cooperative (Pearson test statistic=0.001, p-value=0.9754). Weekly income (β =-0.00004, Chi-Squared =0.05, p-value=0.8233) and log-transformed weekly income (β =0.1025, Chi-Squared =0.16, p-value=0.6849) were not significant predictors of AF awareness. The amount of peanuts harvested was also a poor predictor of the log odds of AF awareness (β =-0.0003, Chi-Squared=0.26, p-value=0.6095).

Discussion

Socioeconomic Profile of Participating Peanut Farmers

Our data reveal a socioeconomic context similar to that of previous studies conducted in Haiti. Nearly three-quarters of participants were male, a proportion comparable to Pluviose, who reported that 71% and 74% of coop and non-coop participants, respectively, were male (Pluviose, 1991). The number of household members was higher among our participants compared to the average household size of 4.6 members, as reported by the World Bank (World Bank, 2006). A higher percentage of our participants reported secondary-level education (35%) compared to Pluviose (1991), who found that 3-4% of coop and non-coop peanut producers in the vicinities of Capotille and D' Osmon had secondary-level schooling.

Our examination of land tenure showed a lower prevalence of land ownership (31%) and higher renting (23%) and sharecropping (15%) compared to previous studies. Wiens and Sobrado estimated in 1995 that 32.4% of national

landholdings had been purchased, 33.1% inherited, 8.4% rented, and 11.9% sharecropped (Wiens & Sobrado, 1998). Pluviose (1991) found that 72% and 66% of cooperative and non-cooperative farmers cultivated peanuts only on their own land.

Our income data from farmers should be interpreted very cautiously because of bias due to sampling and reporting error among farmers. Some farmers do not maintain accurate records of their expenses and income; understandably, others are reluctant to reveal them. Notwithstanding, we calculated the geometric mean of individual farmer annual income to be US\$836 (95% CI: US\$675 to 1035), and 50 farmers (51%) reported a weekly income equivalent to less than US\$2.00 per day. Haiti's recently estimated GDP per capita was US\$1,300 (Central Intelligence Agency, 2013), and 80% of Haitians were estimated to live on less than US\$2.00 per day (World Bank, 2013b). The World Bank reported in 2006 that the percent of off-farm income among rural Haitian households ranged from 25.8 to 34.4% for the five income-based quintiles (World Bank, 2006). We confirmed the importance of non-farm employment to supplement farming income, finding that 57% of our farmers depended on non-farm employment. Pluviose (1991) reported that 7% and 29% of non-coop and coop participants, respectively, depended on non-farm income.

In assessing the economic constraints to peanut production and AF control, attention should be drawn to major household expenditures that affect farmers' ability to purchase agricultural inputs that improve production. The average annual amount spent on food (US\$1,456), calculated based on the

weekly reported amount, was the largest expense. For families with the average number of children (3.2) the cost of education (given the average cost of education per child per family, US\$106) was the second greatest expenditure, followed by healthcare. The sum of averages for these household expenses exceeded the total individual farmer income by more than two-fold. The discrepancy between income and expenses is consistent, however, with the observation that 80% of participants reported that other family members contributed to household expenses.

By viewing farmer expenses as relative to one another, our data suggest how farmers prioritize expenditures. Consequently, an increase in the cost of one would affect farmers' ability to manage others. One perennial illustration is the synchrony of Haitian hospital activity and the school year: after families pay for school tuition, uniforms, and books, the number of patients seeking hospital services is visibly reduced. Viewed through this lens, farmers' ability to purchase agricultural technologies— seeds, equipment, agricultural chemicals—is limited, corroborated by our finding that 40% of farmers cited inaccessibility to credit as a major production constraint. Furthermore, farmers without credit are less able to time peanut cultivation and sale to optimize production and profits.

Farmers' Access to Agricultural Technology

Access to agricultural technologies and livestock ownership were consistent with past studies. Comparable to the subjects of Pluviose (1991), our participants had no access to irrigation, pesticides, or mechanized farming equipment. Pluviose also reported that the machete and hoe were the most

important tools. Our data revealed that small-scale livestock ownership observed in other parts of the country applies to peanut farmers in the northeast as well. Haiti's Ministry of Agriculture maintained that 80% of family agricultural production included poultry, 65% goats, 55% cattle, and 45% pork but noted the limited availability of commercial animal feed and veterinary care (Ministry of Agriculture, 2010).

AF Awareness and Exposure to MFK AP

We found a significant and positive association between farmer awareness of AF and proxies for MFK outreach, including attendance of farmer trainings, participation at demonstration plots, and familiarity with MFK agronomists. General familiarity with MFK, however, was independent of AF awareness. That more farmers recalled MFK's agronomists than MFK itself implies the important role the organization's farmer outreach program plays; farmers more readily recalled the practices they learned and the agronomists they met rather than the organization responsible. That farmers shared their knowledge from training marks MFK's broader influence via horizontal transfer that is necessary for large-scale AF management.

AF Awareness and Socioeconomic Indicators

AF awareness did not have a significant association with indicators of farmer wealth and market access, such as cooperative membership, income, and quantity of peanuts harvested. Furthermore, indicators of access to farmer outreach, such as participation of peanut demonstration plots and familiarity with MFK agronomists, were not significantly associated with log income as of July 2011. These results suggest a lack of quality valuation and are

consistent with a market where few incentives encourage farmers to produce low AF peanuts.

Previous studies outside of Haiti reported farmer education to be significantly predictive of AF management practices. Awuah et al (Awuah et al., 2009) showed that Ghanaian farmers' decision to sort peanuts prior to sale was strongly influenced by a range of factors, including education and knowledge of AF and its health effects. Kumar and Popat (G D S Kumar & Popat, 2010) showed a significant and positive association between education and AF management practices among Indian farmers. Yet among farmers in our study, both farmer education and peanut sorting were independent of AF awareness. Determining any association between sorting and AF awareness was a challenge for two reasons: first, our sample was saturated with farmers who reported to sort their peanuts, and second, our survey did not allow farmers to differentiate themselves based on how or why they sort their peanuts. Regarding the non-association between education and AF awareness, the latter was not significantly different between farmers who had schooling at the primary level, secondary level, or had no schooling, suggesting that primary and secondary schooling did not make knowledge of AF more accessible. Because only one farmer had a university education, we could not test whether farmers with education beyond the secondary level were more likely to be aware of AF.

Study Limitations and Future Directions

In considering the feasibility of future evaluations of production and capacity to control AF among Haitian peanut farmers, the limitations of our study must

be taken into consideration. First, we were unable to rigorously obtain information about peanut purchases, such as fluctuation and seasonality of prices and location of seed purchases. Second, more information is needed regarding sorting and storage practices. This includes the criteria by which farmers sort and remove less desirable kernels (i.e. moldy, immature, damaged pod, split seed, etc.) and duration, location, and conditions of storage. Another major challenge was the estimation of income among Haitian farmers. Our study approximates farmer earnings and priorities of expenditures, yet an accurate breakdown of participants' income and expenses remains difficult to estimate.

The greatest limitations were our small sample size and non-random selection of participants. We were unable to randomly sample our participants because of the challenges associated with identifying and locating peanut farmers, none of whom were registered with a formal farming organization. While we have no reason to believe that our data misrepresent peanut farmers, caution should be taken when extrapolating conclusions to peanut farmers throughout Haiti, for instance, in areas outside the catchment of MFK AP and peanut procurement. Future studies should attempt to randomly sample farmers. Upon reaching a village, we recommend that survey teams generate a list of local peanut farmers with village authorities and then randomly choose participants. A more robust attempt at random sampling would improve the scope of possible inference.

Conclusions

Studies have repeatedly shown that Haitian agricultural production and farmer livelihoods are constrained by, among other factors, limited access to agricultural technologies. Using data from a formative study of farmer livelihoods, we characterized the socioeconomic and agronomic context of 109 Haitian peanut farmers and detected significant associations between farmers' awareness of AF and their exposure to MFK AP. We did not detect a significant association between AF awareness and socioeconomic indicators such as reported income, market access, and farmer education. Our profile was consistent with previous reports showing that access to improved seed varieties, agricultural chemicals, and irrigation, and land tenure were limited among all participants. Furthermore, forty percent of farmers in our study cited inaccessibility to credit as a constraint to peanut production. We implore that future studies build on our work, particularly in other peanut producing regions like the Central Plateau and South East Department (US Agency for International Development, 2005). Haitian producers could readily control AF levels in peanut products but can only do so with adequate pre-harvest technologies and sound storage practices. As rural development agencies and the Haitian government seek to expand food safety capacity along the peanut value chain, periodic assessment of farmers' constraints and opportunities to manage AF contamination will prove critical.

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CHAPTER 5: AFLATOXIN CONTAMINATION IN HAITIAN PEANUT PRODUCTS AND MAIZE AND THE SAFETY OF OIL PROCESSED FROM CONTAMINATED PEANUTS¹

Abstract

The primary objective of this study was to monitor aflatoxin contamination in Haitian samples of raw peanuts (n=21), peanut butters (n=32), and maize (n=30) obtained in Port-au-Prince and Cap Haitien, Haiti during 2012 and 2013. Our secondary objective was to explore a process that uses a locally produced Haitian spirit (*clarin*) to transform oil from contaminated peanuts into a safe, edible product. Immuno-affinity column chromatography and fluorometry (VICAM Aflatest) detected aflatoxins in 14%, 97% and 30% of raw peanuts, peanut butters, and maize samples, respectively, and the concentration of total aflatoxins was greatest in peanut butters (median: 137 µg/kg, maximum: 2720 µg/kg). The concentration of aflatoxin in extracted oil was on average 10% of that in un-extracted oil which, in turn, had a concentration that was only 5% of the original contaminated peanuts. Therefore, aflatoxin concentration in the final product was 99.5% less than that found in the original peanuts, even without pre-filtration. Our extraction experiments testing laboratory-grade ethanol and *clarin* provide evidence that the latter can serve as a low-cost alternative to effectively reduce aflatoxin concentrations in oil pressed from high aflatoxin peanuts.

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Introduction

Aflatoxins are toxic secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus* and include a stable and highly oxygenated structure of 5 fused rings and a lactone moiety. Aflatoxin B₁, the most abundant of aflatoxins, is a causative agent in hepatocellular carcinoma and is associated with immune-dysfunction and protein deficiency syndromes such as kwashiorkor (Coulter et al., 1986; Turner, Moore, Hall, Prentice, & Wild, 2003; Wogan, 1992).

Though found in a range of crops, including spices (Hammami et al., 2014), tree nuts (Georgiadou, Dimou, & Yanniotis, 2012), maize and peanuts (Jager, Tedesco, Souto, & Oliveira, 2013), aflatoxin contamination is most prevalent in the latter two, occurring both before and after harvest (Williams et al., 2004). When stressed by drought and pest pressure, maize and peanuts are most prone to infection by toxigenic *Aspergillus* and contamination with aflatoxin (Pitt, Taniwaki, & Cole, 2013). In addition, warm, humid storage conditions result in post-harvest fungal growth and increased aflatoxin concentrations (Turner et al., 2005). Consequently, aflatoxins are often detected in foods from tropical countries where irrigation and pest management practices are lacking and food storage is poor (Williams et al., 2004).

In Haiti, exposure to aflatoxins, as indicated by circulating blood-albumin was detected among outpatients residing in Port-au-Prince (Schwartzbord et al., 2014), and aflatoxin contamination has been documented for Haitian maize (Castor, Mirocha, & Chang, 1987) and peanut butters (Filbert & Brown, 2012). Filbert and Brown collected peanut butter samples in Port-au-Prince during December of 2009 and October 2010 (Filbert & Brown, 2012). Using immuno-affinity column chromatography coupled with fluorometric detection, they

found that aflatoxin levels ranged from 7.9 to 799.8 µg/kg aflatoxin, and 16 out of 18 samples had more than 20 µg/kg, the US Food and Drug Administration (FDA) regulatory limit. Aflatoxin contamination in Haitian maize, a staple crop, has been described in one peer-reviewed study (Castor et al., 1987). Castor's team collected maize samples from markets during January, July, and October of 1983 and January of 1984, and total aflatoxins were measured using HPLC. Twenty-two percent of the 268 samples had greater than 20 µg/kg aflatoxin.

A primary motivation for examining aflatoxin in the food supply of a resource-limited country is to explore feasible processes that will attenuate contamination to acceptable levels. Removal of contaminated kernels by visual, tactile and density segregation are examples of effective physical separation and are feasible among Haitian food processors (Filbert & Brown, 2012). In places where poverty is pervasive, however, the subsequent challenge after separating contaminated kernels is that they will be discarded by the processor but obtained in local, unregulated markets and construed as edible among food insecure individuals (Matumba, Van Poucke, Monjerezi, Njumbe Ediage, & De Saeger, 2015). Pursuant to minimizing aflatoxin exposure among the poorest of consumers, it is essential to prevent highly contaminated kernels from reentering food chains, and decontamination of such kernels should complement sorting practices. Many chemical decontamination processes exist and are reviewed extensively by Leibetseder (Leibetseder, 2006), including treatments to lessen the potency of the aflatoxin molecule and solvent extraction techniques. Chemical treatment with ammonia (Weng, Martinez, & Park, 1994) and oxidizing agents such as ozone

(Luo, Wang, Wang, Li, Bian, et al., 2014; Luo, Wang, Wang, Li, Wang, et al., 2014), for instance, have been shown to reduce aflatoxin concentration to 1-36% and 11-13% original levels, respectively, depending on treatment parameters. Also effective is solvent extraction using aqueous ethanol, which reduces aflatoxins to 2-7% original levels in cottonseed and peanut meals (Rayner, Dollear, & Codifer, 1970). Ammoniation, ozone treatment, and ethanol extraction are not equally feasible in lesser-developed countries. Anhydrous ammonia and ozone, for instance, are often not readily available in lesser-developed countries such as Haiti. Furthermore, residual ammonium following ammonia treatment is not permissible in human food, and ozone hastens lipid peroxidation in oil seeds. Imported ethanol and locally produced ethanol, however, are available in Haiti, the latter being less costly and more economically accessible to small-scale peanut processors.

Given that Filbert and Castor *et al* reported their results in 2012 and 1987, respectively, the primary purpose of our study was to monitor more recent aflatoxin contamination of Haitian peanuts and locally produced maize during 2012 and 2013. As a corollary, we sought to identify a safe, value-added product made from formerly aflatoxin-contaminated peanuts, and we considered production of edible oil as potentially suitable to Haitian food processors. Therefore our secondary purpose was to determine aflatoxin carryover from contaminated kernels to un-refined, edible oil and the efficacy of extraction, comparing both laboratory-grade ethanol and a locally procured Haitian spirit, on the residual aflatoxin concentrations found in such oil. This comparison was made because extraction using a local ethanol would be more

feasible among small-scale food processors, who produce the majority of peanut butter sold in Haiti.

Materials & Methods

Food Samples

Samples were collected during three periods. During July of 2012, 14 peanut butters and 21 peanut samples were obtained from open-air markets in Port-au-Prince and Cap Haitien. In December 10 maize samples of approximately 1.0 kg each were obtained from the Telele, Croix du Bouquets, and Croix du Bosales markets in Port-au-Prince. The third period was September through December of 2013, during which 21 peanut butters were purchased around Cap Haitien, and 20 maize samples were obtained at four farmer association depots and three mills in the Nord Department.¹ At depots, where farmers sort and grade the maize as fit for human consumption or animal feed, representative samples were taken and kept separate based on classifications described by farmers onsite. Whole, sound ears of corn were generally directed to the mill, while those with visible rot and free kernels on the ground were directed to animal feed. At mills, a sampling probe was used to obtain kernels from the bottom, middle, and upper parts of storage sacks weighing 50 to 100 kg. Milled maize was sampled where available and included grain for maize porridge (“mayi moulén”), fine maize flour (“mayi farin”), and bran destined for animal feed (“mayi pay”). Of the 20 maize samples collected, 11 were directed to human consumption but not yet milled, 5 were directed to human consumption and milled, and 4 were directed to livestock feed and included milled and non-milled maize. Each sample

¹ Mill operators granted access for sampling on the condition that the locations remain anonymous.

weighed 1.5-2.0 kg and was taken from a 50-150 kg storage sack. Moisture for whole grain samples was measured the day of collection. Samples were stored at -30°C until milling with a hammer-mill.

Determination of Aflatoxin with Immuno-Affinity Column Chromatography and Fluorometric Detection

Aflatoxin was measured using the VICAM Aflatest system (Journal AOAC, 17th edition, 2000, 972.26). Each peanut butter was emptied from its original jar and mixed thoroughly with a spatula before sub-samples (25 g) were taken for analysis. Peanut samples were ground with a small food processor prior to sub-sampling. Maize samples included whole cobs (10-15) and free kernels (1.5-2.0 kg) obtained from farmers and mill depots. Kernels from whole cobs were removed by hand, and each sample was ground in a hammer mill with a 4.0 mm screen. Sub-samples (50 g) were taken for analysis. Aflatoxin was extracted from samples (60% or 80% methanol for peanut or maize samples, respectively) using a blender at high speed for 1 minute. Extract was filtered with fluted filter paper (VICAM) into a glass beaker and diluted with de-ionized water (1:2 and 1:5 dilutions for peanut-products and maize, respectively), followed by filtration with a glass microfiber filter. Dilute filtrates for peanut (10 ml or 1.0 g sample equivalent) and maize (2 ml or 0.2 g equivalent) were passed through an immune-affinity column. For samples outside the range of detection (0-100 µg/kg and 0-300 µg/kg for peanut and maize products, respectively) filtrate was further diluted 1:5 (10 ml dilute filtrate and 40 ml de-ionized water) or 1:10 (5 ml dilute filtrate and 45 ml de-ionized water). The column was washed with de-ionized water twice and eluted into a borosilicate culture tube with 1.0 ml of HPLC-grade methanol. To

the eluate was added 1.0 ml of brominated water, and the sample was vortexed. After 1.0 minute, fluorescence of the sample was read (Excitation: 360 nm, Emission: 440 nm). Maize reference samples with no detectable aflatoxins, 50.8, 9.6, 5.9, and 1.7 $\mu\text{g}/\text{kg}$ were obtained (Trilogy Labs, Washington, Missouri), and peanuts butters with no detectable aflatoxins were spiked with 1, 2, 3, 4, 10, 25, and 400 $\mu\text{g}/\text{kg}$. The spiking standard (Trilogy Labs) contained 5 $\mu\text{g}/\text{ml}$ total aflatoxins (2 μg AFB1, 2 μg AFG1, 0.5 μg AFB2, and 0.5 μg AFG2 per ml acetonitrile). All reference samples were assayed in triplicate, and the limit of detection (LOD), limit of quantitation (LOQ), recovery range %, and relative standard deviation (RSD %) were determined. We set our LOD to the lowest reference sample whose mean result was significantly different (Student's t-test, $p < 0.05$) from that of the reference without additional aflatoxins and our LOQ to the level with an acceptable recovery and a relative standard deviation of 25% or less. Raw peanut, peanut butter, and maize samples obtained in Haiti were assayed in duplicate.

Safety and Efficacy of Oil as an Alternative Use of Contaminated Peanuts for Haiti

A Kern Kraft Oil Prince 20F Screw Press (manufactured by Screw-Press GmbH, Germany) was used to produce oil from naturally contaminated peanuts that had been manually sorted. Five separate 10 kg batches of peanuts had aflatoxin concentrations ranging from 155 to 30,000 $\mu\text{g}/\text{kg}$ (median=13000 $\mu\text{g}/\text{kg}$) and were pressed with the oil seed expeller. Following aflatoxin analysis of oil and press cake samples, we evaluated aflatoxin extraction from those oils using ethanol-based solvents in three experiments.

Oil was extracted with a Haitian spirit containing 50% ethanol (*Clarín*, CL) in two experiments. For the first, two naturally contaminated oils were extracted, one with a beginning aflatoxin concentration of 185 µg/kg (High aflatoxin oil, HA) and the other containing 19 µg/kg total aflatoxins (Low aflatoxin oil, LA). A 50 g aliquot of each was treated with 250 ml CL or 150 ml CL and 10 g NaCl. HA and LA were pressed from peanuts that originally had aflatoxin concentrations of 18200 µg/kg and 1160 µg/kg, respectively. In the second experiment with CL, naturally contaminated peanut oil with 33 µg/kg total aflatoxins was mixed with CL at peanut oil: solvent (g:ml) ratios of 1:3, 1:2, 1:1, 5:3, and 5:1. Briefly, 50 g aliquots of peanut oil and 5 g salt were mixed with 150, 100, 50, 30, or 10 ml of the extraction solvent.

In our third extraction experiment, we treated an oil containing 152 µg/kg total aflatoxins using 50% ethanol solution prepared from HPLC grade ethanol. The oil : solvent ratios (g:ml) of 1:3, 1:2, 1:1, 5:3, and 5:1 were used. For all extraction experiments, the oil and solvent were hand shaken for one min. A clean phase separation was observed following approximately 10 min, after which 25 g of oil were removed for aflatoxin analysis.

Results and Discussion

Method Quality Assurance and Occurrence of Aflatoxins

The quality assurance of our method is summarized in **Table 5.1**. Average recoveries (relative standard deviation in parentheses) for peanut butters spiked with 2, 3, 4, 10, 25, and 400 µg/kg were 110 (65), 74 (65), 86 (7.3), 90 (14), 76 (11), and 89% (3.2%RSD), respectively. For maize with 5.9, 9.6, and 50.8 µg/kg, recoveries and variation were 49 (10), 67 (7.0), and 73% (4.1 %RSD). As

the peanut butter without additional aflatoxin measured 0.01 µg/kg, the LOD for peanut butter was 2 µg/kg ($p=0.0209$). The LOD for maize was 5.9 µg/kg ($p=0.0115$).

Table 5.1: Quality assurance of methods to measure total aflatoxins in peanut butter and maize by immuno-affinity column chromatography with fluorescence detection.

Analyte	Matrix	LOD ^a (µg/kg)	LOQ ^b (µg/kg)	Recovery Range%	RSD _r % ^c
Aflatoxins	Peanut Butter	2	4	76-90	3-14
Aflatoxins	Ground Maize	5.9	9.6	67-73	4

^a The limit of detection (LOD) was based on the lowest reference sample with a mean aflatoxin concentration significantly greater (student's t-test, $p<0.05$) than that of the non-spiked reference.

^b The limit of quantitation (LOQ) was based on an acceptable recovery range and a relative standard deviation (RSD_r) less than 25%.

^c All reference samples were tested in triplicate.

Table 5.2: Incidence of occurrence of total aflatoxins. ^a

Product	% Positive ^b	Total Aflatoxins (µg/kg)		Number of samples in each concentration range					Total Samples
		Median	Range	<LOD ^c	LOD-20 (µg/kg)	20-100 (µg/kg)	100-1000 (µg/kg)	>1000 (µg/kg)	
Raw peanuts- Port-au-Prince, 2012	14	< 2.0	<2.0-787	18	0	2	1	0	21
Peanut Butters- Port-au-Prince, 2012	91	137	<2.0-2720	1	1	3	4	2	11
Peanut Butters- Cap Haitien, 2013	100	335	28- 1850	0	0	2	17	2	21
Port-au-Prince- Ground Maize, 2012	0	<5.9	<5.9	10	0	0	0	0	10
ND Maize- All, 2013	30	<5.9	<5.9-78	14	5	1	0	0	20
ND Maize- Not milled, 2013	27	2.855	<5.9-78	8	2	1	0	0	11
ND Maize- Milled for Porridge and Flour, 2013	20	<5.9	<5.9-9.85	4	1	0	0	0	5
ND Maize for Livestock-Milled and Non-Milled, 2013	50	7.025	<5.9-10.65	2	2	0	0	0	4

^a Total aflatoxins were measured by immuno-affinity column chromatography and fluorometry in peanut butters, raw peanuts, and maize obtained in Port-au-Prince and the Nord Department of Haiti (ND), including Cap Haitien.

^b The % Positive refers to samples greater than the limit of detection (LOD).

^c The LODs were 2 and 5.9 µg/kg for peanut butters and maize, respectively.

The incidence of occurrence of aflatoxins in raw peanuts, peanut butter, and maize are in **Table 5.2**. The median of total aflatoxins was greatest in peanut butters, followed by ND maize and raw peanuts. Peanut butters had the greatest incidence of aflatoxin contamination (94% of samples with 20 µg/kg or greater), followed by raw peanuts (14%) and ND maize (5%). The maize market samples obtained in Port-au-Prince had no detectable aflatoxins.

Our results are consistent with those of previous studies on aflatoxin contamination in Haitian maize and peanut products. Filbert and Brown measured a maximum of 799.8 µg/kg in the samples they obtained in 2009 and 2010 and found that 89% were in excess of the FDA Action Level of 20 µg/kg. The incidence we found of peanut butters three years later with greater than 20 µg/kg total aflatoxins was comparable to that of Filbert and Brown, though 4 of the peanuts butters we tested (13%) had greater than 1000 µg/kg total aflatoxins. The present study found maize aflatoxin contamination greater than 20 µg/kg in 5% of 2012-2013 samples, while that of Castor et al (1987) found 22% of 1983-1984 samples with that level of contamination.

In this comparison, a picture emerges in which aflatoxin contamination of Haitian peanut butters is more prevalent than that of locally procured maize. This difference in contamination may be a product, to some extent, of post-harvest practices among peanut producers and processors. Previous studies have shown that aflatoxin contamination is reduced by post-harvest processing, such as sorting of moldy kernels and adequate storage practices (Filbert & Brown, 2012; Hell, Cardwell, & Setamou, 2000; Kaaya, Harris, & Eigel, 2006). In Haiti, post-harvest processing and storage of maize and

peanuts differ. Peanut producers typically store peanuts in nylon or natural fiber sacks on earthen or concrete floor indoors. Though peanut processors remove kernels with visible mold from snacking peanuts, sorting is uncommon prior to peanut butter production. In contrast, maize farmers dry and store whole ears with husks in trees for up to six months at a time, a practice that occurs in many lesser-developed countries (Udoh, Cardwell, & Ikotun, 2000). Kernels and whole ears without husks are stored in nylon bags and in an open pile on the ground, respectively, at home or in small cooperative depots for shorter periods. Farmers who we met reported that free kernels on the floor of cooperative depots and ears of maize with rot are not milled but reserved for animal feed, and processors discussed removal of damaged kernels prior to milling. Sorting and removal of contaminated peanut kernels is effective and will be essential to managing aflatoxin contamination in Haiti (Filbert & Brown, 2012; Kaaya et al., 2006).

Yet a major concern is that the removal and consequent concentration of aflatoxin in the rejected peanuts will create a supply of dangerous material that looks like food. There is a real risk and some evidence that the poorest and most food insecure individuals will scavenge or purchase rejected peanuts and become exposed to foods that are much more contaminated and toxic than the unsorted peanuts (Williams et al., 2004).

Safety and Efficacy of Peanut Oil Processing

Clearly, peanuts rejected due to aflatoxin contamination need to be diverted from direct human consumption to uses that reduce the magnitude of aflatoxin exposure below what was present with the unsorted supply

(Matumba et al., 2015). One such alternative use is to press oil from the rejected peanuts and strip the oil of the small amount of aflatoxin that may be carried away in the oil by particulates or emulsion. Such a product may preserve enough of the market value of the rejected peanuts to prevent their sale for human consumption, yet reduce the amount of aflatoxin streaming into the human food chain. Edible oil is certainly a product appropriate to existing value chains in Haiti and other lesser-developed countries. Our study evaluated the carryover of aflatoxins from contaminated kernels to crude oil to ethanol-treated oil, and we chose to compare the extraction efficacy of an HPLC-grade ethanol and a locally made spirit (CL) because the latter would lower the cost and increase the feasibility of aflatoxin extraction among Haitian peanut processors.

Aflatoxin concentration ($\mu\text{g}/\text{kg}$) in oil pressed from batches of contaminated peanuts ($n=5$) was reduced to a mean of 5.0% of the original concentration in the roasted kernels [maximum=12%, minimum of original concentration= 1%, standard error of the mean (SEM)= 2.2%]. The original sample that was least contaminated with aflatoxin was a mix of peanut germ and skins removed from kernels at a Haitian peanut processing facility. Total aflatoxins in the germ-skin mix, press cake, and corresponding oil were 155, 99, and 19 $\mu\text{g}/\text{kg}$, respectively.

The results from the first extraction experiment are shown in **Table 5.3**, and the findings of the second and third are in **Table 5.4**. Taking together the results of all three, aflatoxin was reduced to a mean of 10% (standard error of the mean=2.2%) of the original oil using an oil: solvent ratio of 1:3 in triplicate.

In the case of HA, total aflatoxins were reduced from 18200 µg/kg whole peanuts to 185 µg/kg pressed oil to 11 µg/kg CL-treated oil. The comparison of experiments 2 and 3 should be interpreted with caution, as the initial aflatoxin concentration in the two treatment groups differed.

Notwithstanding, we observed a reduction in aflatoxin that followed a comparable dose-response relationship using both CL and 50% HPLC ethanol, the former of which would be more economically accessible to Haitian peanut processors.

Table 5.3: Extraction of aflatoxin from two peanut oils using a Haitian spirit containing 50% ethanol.

	HA ^a		LA ^b	
Peanut oil: <i>clarin</i> ratio (g:ml) ^c	1:5	1:3	1:5	1:3
Aflatoxin concentration following extraction (µg/kg) ^d	13	11	1.4	2.5
Reduction in oil aflatoxin concentration (%)	93	94	93	87

^a High aflatoxin oil (HA) originally had 185 µg of total aflatoxins/kg oil.

^b Low aflatoxin oil (LA) originally had 19 µg total aflatoxins/kg oil.

^c Oils were shaken with salt and *clarin*, a Haitian spirit containing 50% ethanol.

^d Total aflatoxins were quantified using immuno-affinity column chromatography with fluorescence detection.

Table 5.4: Aflatoxin extraction from oil using *clarin* or 50% HPLC ethanol. ^a

Peanut oil:extraction solution ratio (g:ml) ^b	Aflatoxins remaining in 33 µg/kg oil after extraction with <i>clarin</i> (µg/kg) ^a	Reduction of aflatoxin concentration in 33 µg/kg oil (%)	Aflatoxins remaining in 152 µg/kg oil after extraction with 50% HPLC ethanol (µg/kg)	Reduction of aflatoxin concentration in 152 µg/kg oil (%)
1:3	3.8	88	15	90
1:2	4.1	88	14	91
1:1	5.2	84	26	83
5:3	6.8	79	41	73
5:1	14	58	87	43

^a *Clarin* is a Haitian spirit containing 50% ethanol.

^b Aliquots of two oils containing 33 µg/kg and 152 µg/kg total aflatoxins were shaken with *clarin* and 50% HPLC ethanol, respectively, at 5 different oil:solvent ratios.

Our finding underscores that future studies further examine the comparative efficacy and socio-economic factors that would influence adoption of locally procured spirits to extract aflatoxins in resource-limited contexts similar to rural Haiti.

Conclusions

This study showed that 94% of peanut butters obtained in Port-au-Prince and Cap Haitien, Haiti, during 2012 and 2013 had greater than 20 µg/kg total aflatoxins. Of whole peanut and maize samples from the Nord Department, in contrast, 14% and 5%, respectively, were contaminated in excess of that FDA regulatory limit. These results further implicate Haitian peanuts and maize, particularly peanut butters, as sources of aflatoxin exposure among the Haitian people. Farmers and food processors in lesser-developed countries such as Haiti need safe and efficacious alternative uses of contaminated foods, and we examined the safety of ethanol extraction to reduce aflatoxin in contaminated peanut oil. This study showed that CL and 50% HPLC-grade ethanol were comparable at extracting aflatoxins from the oil of highly contaminated peanuts. Aflatoxins were reduced by up to 94% using CL, a Haitian spirit containing 50% ethanol that is obtainable even in remote regions of Haiti where laboratory-grade solvents are unavailable or too costly. After oil pressing and extraction, our data indicate that, on average across a broad range of contamination rates, peanut oil can be produced with only 0.5% of the aflatoxin concentrations found in the peanuts before pressing. Our approach could be one of multiple practices to re-direct contaminated foods to a safe, alternative product in resource-limited countries. In areas where aflatoxin contamination is high and poverty is widespread, further exploration

of simple and accessible alternative uses of tainted foods will be essential in limiting exposure to aflatoxin.

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PART 3

CHAPTER 6: AFLATOXIN-LYSINE ADDUCTS IN HAITIAN PATIENTS INGESTING PEANUT AND MAIZE PRODUCTS¹

Abstract

Aflatoxins are mycotoxins mainly produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. As the major contaminants of peanuts and maize, aflatoxins are causative agents of liver cancer and are associated with immune dysfunction, stunting, and protein deficiency syndromes. Aflatoxins are known to contaminate maize and peanut-based foods in Haiti. Patients at GHESKIO clinic in Port-au-Prince, Haiti, provided blood samples and participated in a dietary survey. Blood samples were analyzed for aflatoxin covalently bound to blood albumin through lysine. Data were analyzed using nominal logistic models, least-squares regression, and ANOVA. Detection of AFB₁-lysine above 0.25 pg AFB₁-lysine/mg albumin was dependent upon frequency of peanut consumption ($p < 0.0486$) (FP) but not frequency of maize consumption (FM), and a nominal logistic model demonstrated that detection was positively associated with FP. In a least-squares regression model, the effect of FP was significantly predictive of log-transformed AFB₁-lysine above 0.25 pg/mg. All 12 of the individuals with detectible circulating aflatoxin biomarkers who had not eaten peanuts had eaten maize or maize products.

¹ This chapter, with modification, was previously published as: Schwartzbord, J. R., Brown, D. L., Pape, J., Verdier, R., Filbert, M. E., & Wang, J. S. (2014). Aflatoxin-Lysine Adducts in Haitian Patients Ingesting Peanut and Maize Products. *Journal of Hunger & Environmental Nutrition*, 9(2), 244-255.

Peanuts were not the sole source of aflatoxins in diet of these individuals and maize cannot be ruled out as a contributor of dietary aflatoxin in Haiti.

Introduction

Aflatoxins contaminate staple foods, such as peanuts and maize, in environments where hot, humid climates combined with poor food storage facilitate fungal growth (Cole, Sanders, Hill, & Blankenship, 1985; Dickens, 1977). Aflatoxins consist of a coumarin structure fused to cyclopentone and bi-furan rings and are mainly produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* (Wogan, 1966). These fungi produce aflatoxins between the temperature range of 24 and 35 °C, favored by climates within 40° north and south of the equator (Williams et al., 2004). Within this broad region, the risk of aflatoxin contamination is heightened in places where food safety regulations are nonexistent and where food producers and processors lack capacity to prevent contamination.

The poorest country in the western hemisphere, Haiti, is one such place. Populated with 9.9 million people, it is a Caribbean nation on the western third of the island of Hispaniola, shared with the Dominican Republic (Central Intelligence Agency, 2013). The Haitian government does not regulate food safety, and peanut and maize farmers lack access to agricultural technologies and adequate storage facilities (US Agency for International Development, 2005). Aflatoxins are known to contaminate Haitian peanut-based products. For example, Filbert and Brown sampled Haitian peanut butters in 2010 and confirmed that 8 out of the 10 samples were contaminated with greater than 20 parts per billion (ppb) aflatoxins, the action level stipulated by the US

FDA(Filbert & Brown, 2012). The most contaminated sample had 799.8 ppb aflatoxins. Unknown is the extent of maize contamination.

The public health implications of both acute and chronic aflatoxin exposure are serious. Ingestion of high doses of aflatoxins may cause acute aflatoxicosis, resulting in liver failure or death. The largest and most recent epidemic of acute aflatoxicosis occurred in Kenya in 2004, involving 317 cases with 125 deaths (CDC (Centers for Disease Control and Prevention), 2004). Chronic dietary exposure of moderate to low levels of aflatoxins stunts growth, is associated with protein deficiency syndrome Kwashiorkor, and leads to liver cancer (International Agency for Research on Cancer, 2010; Coulter et al., 1986; Gong et al., 2003). Turner *et al.* found that Gambian children with detectable blood aflatoxin-lysine adducts had lower levels of secretory immunoglobulin A, implicating aflatoxins in immune suppression (Turner, Moore, Hall, Prentice, & Wild, 2003).

Bloodstream aflatoxin-albumin adducts (as indicated by the aflatoxin-lysine adducts found after careful degradation of circulating albumin) are a useful index of aflatoxin ingestion over an extended period of time and have been used to monitor aflatoxin exposure in Ghana, Kenya, the Gambia, China, Togo and Benin (E Azziz-Baumgartner et al., 2005; Gong et al., 2003; Jiang et al., 2005; Peng et al., 2007; Shuaib et al., 2010; Sun et al. 2011; Turner et al., 2002). To date, no study of aflatoxin biomarker analysis among Haitians has been published. Working in Haiti, our objectives were to: 1) Determine the prevalence of aflatoxin bound through lysine to circulating blood albumin in clients presenting themselves to a health clinic in Port au Prince: Le Groupe

Haitien d'Etude du Sarcome de Kaposi et des Infections Opportunités (GHESKIO); 2) Determine if recent maize and/or peanut consumption patterns were related to circulating lysine-bound aflatoxin.

Methods

Sample Collection. Blood samples were taken by venipuncture from 178 clients presenting themselves to the GHESKIO clinic in Port-au-Prince, Haiti. Upon enrollment, GHESKIO staff administered a dietary questionnaire (in French or Haitian Creole) through in-person interviews. Clients were asked how frequently they recalled eating peanut or maize products per week and how long it had been since they had eaten peanuts or maize. Venous serum was collected and stored frozen (-20 °C) until transport to the Wang lab in the United States (University of Georgia, Athens, Georgia).

AFB₁-Lysine Analyses. Serum AFB₁-lysine adduct levels were measured by a modified high-performance liquid chromatography fluorescence (HPLC-fl) method (Qian et al., 2009; 2013). In brief, serum samples (150 µl) were assayed for concentration of albumin and total protein, then digested by Pronase (Calbiochem, San Diego, CA) for 3 hours at 37 °C. The digests were loaded onto a Waters Oasis Max cartridge (Milford, MA). Cartridges were sequentially washed and eluted with 2% formic acid in methanol. The eluents were evaporated to dryness and reconstituted in 150 µl of 10% methanol prior to HPLC injection. Analysis was carried out on a 1100 liquid chromatography system (Agilent Technologies, Wilmington, DE), and chromatographic separation was performed on a 250 x 4.6 mm Agilent C18 column, for

authentic AFB₁-lysine adduct standard and samples were co-eluted at retention times averaging 12.7 minutes. The detection limit of this method was 10 pg/ml or 0.25 pg/mg albumin. Results were adjusted for serum albumin concentration and presented as pg AFB₁-lysine/mg albumin, or pg/mg.

Statistical Analyses. Comparing responses to the dietary survey and demographic data, such as age and sex, we sought to determine which independent factors were associated with serum AFB₁-lysine level. Based on the survey, clients were assigned to dietary groups based on frequency of peanut and maize consumption (i.e. 1, 3-4, or 7 days of consumption/week). Aflatoxin-lysine concentrations were also classified by days since peanut and maize consumption (i.e. >14, 7-13, 5-6, 3-4, and 1-2 days ago). A chi-square Pearson test (SAS Institute Inc., 2010) was used to determine dependence or independence between AFB₁-lysine detection and dietary groups. Additionally, a nominal logistic model was created to determine which independent factors, including diet, age, and sex, were significantly predictive of AFB₁-lysine detection as a binary variable (i.e. below or above the detection limit of 0.25 pg AFB₁-lysine/mg albumin):

$$\text{Log}[(\pi_{ij})/(1-\pi_{ij})]=\beta_0+\beta_{ij}x_{ij},$$

where $\text{Log}[(\pi_{ij})/(1-\pi_{ij})]$ refers to the log odds of AFB₁-lys detection, β_0 the y-intercept, and β_{ij} the partial slope of independent variable i at level j . X_{ij} refers to “yes” or “no” for independent variable i at level j .

General linear models with F-ratio effects tests were used to evaluate the significance of independent variables on biomarker values above 0.25 pg/mg as a continuous variable:

$$Y_{ij} = \beta_0 + \beta_{ij}x_{ij},$$

where Y_{ij} refers to predicted natural log of AFB₁-lys, β_0 refers to the y-intercept, and β_{ij} refers to partial slope for independent variable i at level j . X_{ij} refers to “yes” or “no” for independent variable i at level k .

Using analysis of the variance, natural log-transformed AFB₁-lysine levels for all patients were compared between groups based on survey responses. F-ratio tests were conducted and means were compared using Tukey-Kramer HSD ($\alpha=0.05$), with JMP 9 software.

Results

Food Frequency. Of the participating clients at GHESKIO, gender and age were recorded for 174 patients. Females totaled 105 patients, and males 69. Sex was not recorded for 4 patients. Mean age was 35 years (SD= 11 years, median=33, minimum=16 years, maximum=68). Means for frequency of (FP) and days since (DP) peanut consumption and frequency of (FM) and days since (DM) maize consumption were found. The mean value was 1.4 days (SD=0.90) for FP, 2.3 days (SD=1.5) for DP, 1.3 days (SD=0.71) for FM , and 2.3 days (SD=1.4) for DM. **Tables 6.1** and **6.2** show the distribution of responses to dietary survey.

Table 6.1: Frequency of maize and peanuts consumption and distribution of participating clients (% of total clients) at GHESKIO clinic.

Frequency (days/week)	Maize	Peanuts
1	107 (60%)	95 (53%)
3-4	47 (27%)	35(20%)
7	13 (7%)	33 (19%)
No Consumption	11 (6%)	15 (8%)

Table 6.2: Days since consumption of maize and peanuts and distribution of participating clients (% of total clients) at GHESKIO clinic.

Days since Consumption	Maize	Peanuts
>14	53 (30%)	66 (37%)
7-13	50 (28%)	30 (17%)
5-6	33(19%)	32 (18%)
3-4	14 (8%)	11 (6%)
1-2	20 (11%)	28 (16%)
No Consumption	8 (4%)	11 (6%)

AFB₁-Lysine adduct level. Mean AFB₁-lysine for the 178 GHESKIO clients was 3.98 (SE=0.8756) pg AFB₁-lysine/mg albumin, and the median, minimum and maximum were 1.115, 0.3100, and 130.4 pg AFB₁-lysine/mg albumin, respectively. Excluding values below the detection limit (0.25 pg/mg) the mean, median and standard error were 5.174, 2.06, and 1.136 pg/mg, respectively. Two extreme values were noted (client 33 with 69.33 pg AFB₁-lysine/mg albumin and client 139 with 130.39 pg AFB₁-lysine/mg albumin). Client 33 reported FP to be 3-4 times/wk, and DP 3-4 days prior. Her FM was 3-4 times/wk, and DM 1-2 days prior. Client 139 stated FP to be 3-4 times/wk, with his most previous consumption more than 14 d prior to sampling. His reported FM was 3-4 times/wk, and DM 1-2 days prior. The log AFB₁-lysine

level of these two individuals suggest that they either were exposed to an unusually high level of aflatoxin during prior consumption of maize, or alternatively, an even higher level during most recent consumption of peanuts. To determine whether the data of clients 33 and 139 were influential, we calculated the Cook's D distance values, which were 0.92 and 0.21 for clients 139 and 33, respectively. That the Cook's D distance for client 139 approached 1.0 suggested that it could be an influential point. For the same individual, we log-transformed the value for AFB₁-lysine and calculated Cook's D distance, which was 0.092. We concluded that clients 33 and 139 would not act as influential points in a general linear model of the log-transformed data.

Dependence of Detection of AFB₁-lysine Adduct on Dietary Variables. Detection of AFB₁-lysine was significantly dependent upon FP (Pearson test, $p=0.0486$). See **Table 6.3** for data. Detection of the biomarker proved independent of DP ($p=0.2530$), FM ($p=0.2032$), and DM ($p=0.2480$).

Table 6.3: Dependence of detection of AFB₁-lysine above 0.25 pg/ mg albumin upon frequency of peanut consumption (FP).^a

Frequency of Peanut Consumption (days/week)	Below detection limit (number of clients)	Above detection limit (number of clients)
No Consumption	3	12
1	28	67
3-4	10	25
7	2	31

^a Detection was significantly dependent upon FP ($p<0.0231$).

Binary and General Linear Models. Clients with no detectable and detectable AFB₁-lysine adduct totaled 43 and 135, respectively, resulting in a skewed distribution of the adduct levels. For each model we report the p-value of independent variables based on the effect likelihood ratio test. Prior to proceeding with a general linear model of AFB₁-lysine adduct level, a binary nominal logistic model was constructed to determine which independent factors predicted positivity (i.e. above the detection limit). Interactions between independent variables were tested, too. The initial model included all independent variables, and variables were removed one at a time, starting with the least significant. FP was the single significant independent variable remaining in the model (p=0.0219). No interactions were significant. Data above the detection limit were log-transformed to satisfy normality and used for least-squares regression models. FP was the only significantly predictive variable of log AFB₁-lysine adduct levels (F-ratio=3.7145, p=0.0132 in a single-variable model). There were no significant interactions.

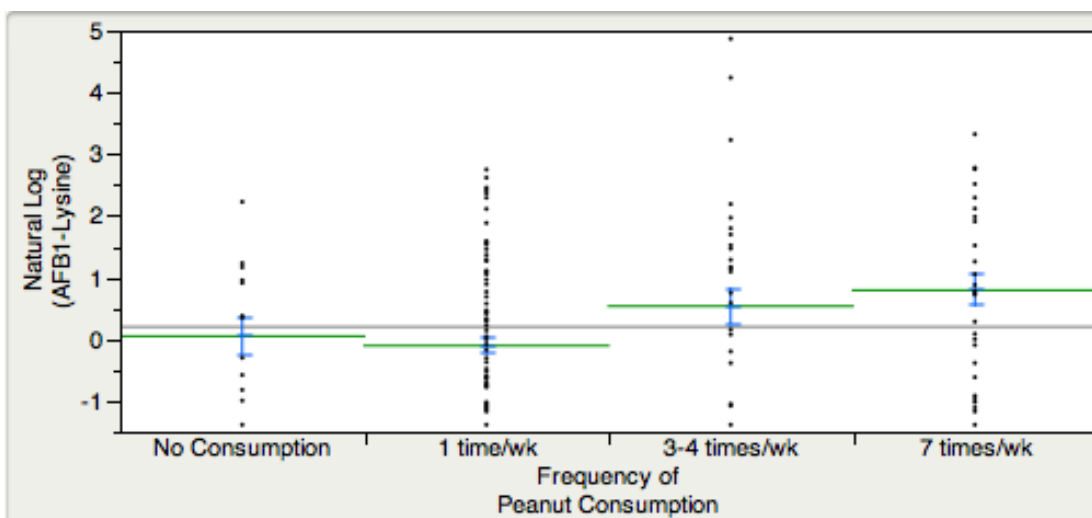
Analysis of the Variance. Mean natural log AFB₁-lysine adduct level was compared among groups based on frequency of peanut or maize consumption. For peanuts, ANOVA analysis had an F-ratio of 4.3396 (p-value=0.0056). Mean log AFB₁-lysine based on peanut consumption is shown in **Figure 1**. For maize, the ANOVA analysis resulted in an F-ratio of 2.2646 (p-value=0.0827). Mean log AFB₁-lysine values and results from the Tukey-Kramer HSD tests are shown in **Table 6.4**.

Table 6.4: Frequency of maize and peanut consumption and mean log AFB1-lysine.^a

Frequency (days/week)	Mean log AFB1-lysine (Standard Error)	
	Peanuts	Maize
No Consumption	0.04927 (0.36) ^{A, B}	0.48657 (0.42) ^A
1	-0.10532 (0.14) ^B	-0.02264 (0.14) ^A
3-4	0.53496 (0.23) ^{A, B}	0.54528 (0.20) ^A
7	0.7943 (0.22) ^A	0.54716 (0.39) ^A

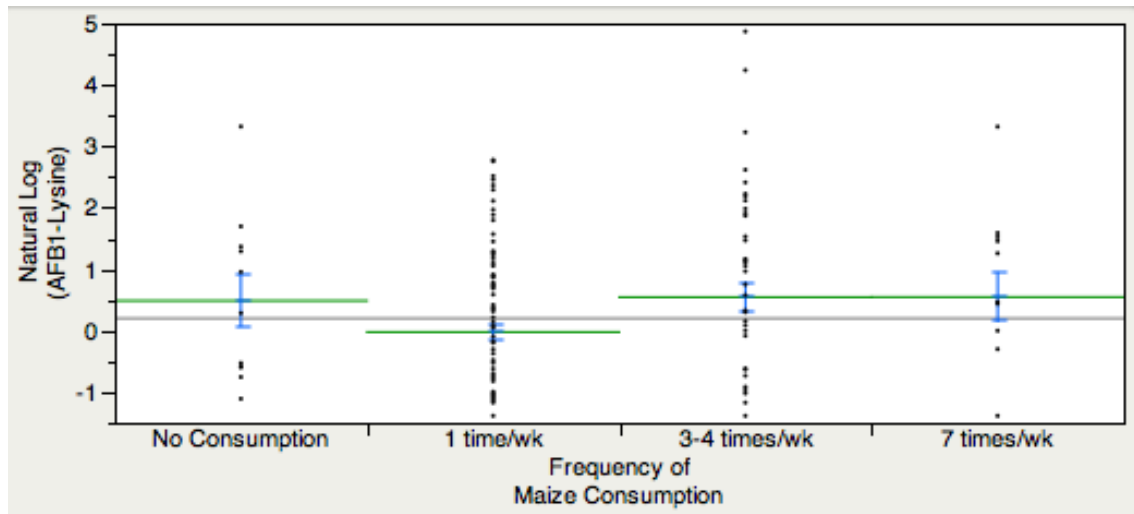
^a Means with different superscripts are significantly different based on Tukey-Kramer HSD

Figure 6.1: One-way analysis of log aflatoxin-lysine adduct level by frequency of peanut consumption ^a



^a Line and error bars indicate group mean and mean errors bars, respectively. The gray bar indicates the grand mean.

Figure 6.2: One-way analysis of log aflatoxin-lysine adduct level by frequency of maize consumption ^a



^a Line and error bars indicate group mean and mean errors bars, respectively. Gray bar indicates the grand mean.

Discussion

In the current study, our purpose was to determine the association between aflatoxin exposure and the consumption of peanuts and maize in Haiti, and we analyzed the effects of independent variables on serum AFB₁-lysine adduct, a reliable aflatoxin exposure biomarker, using logistic and linear models and ANOVA. We found that AFB₁-lysine adduct level above 0.25 pg AFB₁-lysine/mg albumin was significantly associated with frequency of peanut consumption, which is the major dietary factor for human aflatoxin exposure in Haiti. However, our logistic and linear models should be interpreted with some caution. Questionnaire-based food frequency survey is known to be prone to memory mistakes and may explain some recall error in our models. For example, blood samples were mainly obtained from clients who had consumed peanuts and/or maize, but none from those who had

consumed neither. Recalled frequency of peanuts was significantly predictive of log-transformed AFB₁-lysine, but recalled frequency of maize was not. Contrary to our expectations, recalled frequency and days since consumption were not closely associated with each other.

Following analysis of the variance, dietary group means of log-transformed AFB₁-lysine values were compared, and the results of the Tukey-Kramer HSD test showed significant differences between clients who consumed peanuts 1 times/wk and 7 times/wk.

Though the mean of log AFB₁-lysine for non-peanut consumers was lower compared to patients who indicated 3-4 and 7 times/wk consumption, no significant difference was detected between the means of the log-transformed data for those who reported no consumption and 7 times/wk consumption of peanuts. Interpretation of this finding is not without caveat: 12 individuals tested positive for the biomarker and reported no consumption of peanuts, whereas all other peanut consumption groups had greater than 30 individuals. Either the sample size of the non-consumers was too small to detect a significant difference in mean log-AFB₁-lysine, the results were confounded due to memory mistakes, or patients in the non-consumption group had additional dietary sources of aflatoxin. Of patients who reported no consumption of peanuts and tested positive for the aflatoxin biomarker, 2, 9, and 1 patients reported maize consumption of 0, 1, and 3-4 times/wk, respectively. ANOVA demonstrated no detectable difference for mean log AFB₁-lysine among maize consumption groups, though the means for the two groups of greatest maize consumption were greater than those of 0 or 1

time/wk consumption. Of non-maize consumers with detectable AFB₁-lysine, 2, 6, 1, and 2 patients consumed peanuts with a frequency of 0, 1, 3-4, and 7 times/wk, respectively.

Though our study evidenced widespread exposure to aflatoxins, AFB₁-lysine adduct levels of urban Haitians were low relative to instances elsewhere of acute aflatoxicosis. During the 2004 aflatoxicosis epidemic in Kenya, for instance, Azzizz-Baumgartner *et al* found that 0.25 ng AFB₁-lysine/mg albumin was a risk factor (adjusted OR=14.8) for developing acute aflatoxicosis, defined as acute jaundice of unknown origin (Azziz-Baumgartner *et al.*, 2005). None of the patients in our study had an AFB₁-lysine level that met this criterion, for the highest level in this study was 130.4 pg/mg. When Shuaib *et al* (2010) studied birth outcomes of pregnant women for whom AFB₁-lysine was measured, participating Ghanaians with AFB₁-lysine greater than 11.34 pg/mg (considered “very high”) were more likely to have low birth weight babies compared to participants with less than 2.67 pg/mg. In our study, 6% of participants had levels of AFB₁-Lysine greater than 11.34 pg/mg, and 68% less than 2.67 pg/mg.

Important to note, however, is that low-level exposure and detectability of aflatoxin biomarkers are associated with adverse health outcomes such as hepatocellular carcinoma (HCC) and immune dysfunction. Chronic aflatoxin exposure has long been recognized as a causative factor of HCC, the risk of which increases markedly with hepatitis B virus positivity (Kirk *et al.*, 2005; Wogan, 1992). Though the product of multiple factors, Haiti’s HCC incidence rate is worth noting. In their analysis of the International Agency for Research

on Cancer GLOBOCAN database, Phillips *et al* showed that the age-adjusted liver cancer incidence rate in Haiti is the highest in the Caribbean (Phillips *et al.*, 2007). The GLOBOCAN database included the 2002 age-adjusted rates for 26 cancers in 172 countries. The authors compared the rates of 8 cancers for the US and 8 Caribbean countries, including Haiti. Among Haitians, the age-standardized rate for liver cancer incidence was 27.9 per 100,000 males, and for liver cancer mortality 26.8 deaths per 100,000 males. Compared to rates of other cancers in the Caribbean, liver cancer in Haiti had the third highest incidence and second highest mortality rate for men.

The implications of aflatoxin-induced immune dysfunction further merits consideration of chronic, low-dose exposure to aflatoxins. When Turner *et al* measured AFB₁-lysine in Gambian children, the mean was 22.3 pg/mg with a range of 5 to 456 pg/mg. We found 17% of the patients enrolled in our study fell within that same 5 to 456 pg/mg range. Turner *et al* showed that secretory immunoglobulin A decreased from 70.2 µg/mg in children without detectable AFB₁-lysine to 50.4 µg/mg in children with detectable AFB₁-lysine. The implication of immune dysfunction in Haiti is by no means trivial: high incidences of cholera, malaria, tuberculosis, and HIV are well-documented (Agarwal, McMorrow, & Arguin, 2012; Ivers & Walton, 2012; Malow, Rosenberg, Lichtenstein, & Dévieux, 2011; Ocheretina *et al.*, 2012).

Because preventing contamination requires good agronomic practices and proper food storage, aflatoxin is a nexus that links public health, agriculture, and rural development. Actors with an interest in Haiti's health sector should heed the signs of food insecurity for two reasons. First, peanuts and maize

represent important crops to the food security of Haitians, the former being a source of protein less expensive than meat, and the latter an important source of calories. Both peanuts and maize are traditional Haitian foods and are consumed commonly. The FAO estimates that total peanut (in-shell) and maize production in 2010 were 19000 and 233700 metric tons (mt), respectively (Food and Agriculture Organization of the United Nations, 2011). Based on Haiti's 2010 population, per capita productions of in-shell peanuts and maize were 2.0 kg and 24 kg, respectively. Likely representative of consumption, national production of maize and peanuts has surpassed imports by far: Haiti's largest trade partner, the United States, exported 47 mt of maize and 35.9 mt of peanut butter to Haiti in 2011, comparably much less than Haitian national production.

Second, food scarcity is followed with a concomitant increase in the consumption of damaged or contaminated food, often after adverse weather events. This was seen in the case of the 2000-2001 ackee fruit poisoning in Haiti (Moya, 2001). After this event, local public health officials noted that inclement weather had adversely affected food availability in the area of the poisoning and that the victims, mostly children, consumed unripe ackee subsequently (Joskow, Belson, Vesper, Backer, & Rubin, 2006). Over 100 Haitians were acutely poisoned after consuming immature ackee fruit, known to contain elevated levels of two toxins, hypoglycins A and B. Adverse weather was also an important factor in the case of the 2004 aflatoxin poisoning in Kenya: Azzizz-Baumgartner *et al* (2005) maintained that an early rain-season, followed by drought, contributed to elevated aflatoxin contamination and food shortages prior to the poisoning from January to June.

In conclusion, this study measured serum AFB₁-lysine adduct and surveyed recent peanut and maize consumption in 178 Haitian clients at GHESKIO in Port-au-Prince, Haiti. The survey asked clients to recall not only the frequency of consumption but also the number of days since their latest consumption. Our findings indicated a 76% detectable incidence of AFB₁-lysine in this population and a clear association between circulating AFB₁-lysine and the consumption of peanuts. Although how often clients consumed maize was not significantly predictive of the log concentration of AFB₁-lysine, the results from ANOVA suggested a positive association between maize consumption and circulating AFB₁-lysine. Unexpectedly, recalled FP was more predictive of the aflatoxin biomarkers than DP. Assuring the food safety of peanuts and maize is critical to prevent the adverse health effects, both acute and pernicious, of aflatoxins. To do so, future research is necessary to determine the extent of contamination in maize; further characterize aflatoxin exposure, especially in rural areas where maize and peanut consumption likely differ compared to Port-au-Prince; and assess the feasibility of long-term and short-term aflatoxin prevention appropriate for Haitian farmers and food processors.

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CHAPTER 7: DETECTION OF TRACE AFLATOXIN M1 IN HUMAN URINE USING A COMMERCIAL ELISA FOLLOWED BY HPLC

Abstract

Aflatoxin is a liver carcinogen, and rapid, inexpensive methods to detect its urinary biomarkers are needed. We used a commercial enzyme-linked immuno-sorbent assay (ELISA) for aflatoxin M1 in urine (Helica Biosystems) to test 52 Haitian samples. Using this ELISA, we detected traces above the limit of detection (0.2 ng/ml urine), but below the limit of quantitation (0.4 ng/ml) in 14 samples. Liquid chromatography of all 52 Haitian urine samples revealed that only 11 had quantifiable AFM1 (mean: 29.5 pg/ml, standard error: 10.8, range: 2.94-96.5 pg/ml). The Helica ELISA may have detected forms of aflatoxin other than AFM1 in the Haitian samples, or matrix enhancement may have affected results at low AFM1 concentrations. This ELISA may serve as a qualitative indicator of aflatoxin exposure for epidemiological purposes. But this method's utility as a precise and specific indicator of AFM1 concentrations will require additional refinement and validation.

Introduction

Aflatoxins are hepatotoxic mycotoxins produced by *Aspergillus flavus* and *A. parasiticus*, and include aflatoxin B1 (AFB1), AFB2, AFG1, and AFG2. Aflatoxin

contamination is prevalent in certain cereal grains and oilseeds, most notably maize and peanuts, especially where climates favor both growth of the fungi and the production of these toxic secondary metabolites (Williams et al., 2004). In addition to liver cancer, other chronic health effects linked to aflatoxin exposure include immune-dysfunction, stunted growth, and the protein deficiency syndrome, kwashiorkor (Coulter et al., 1986; Gong et al., 2003; Turner et al., 2003; Shuaib et al., 2010).

Urinary and blood-based biomarkers have been used to estimate exposure to aflatoxin B1 (AFB1) in many studies (Turner et al., 2012), and one urinary metabolite of AFB1 that is correlated with intake is AFM1 (Zhu et al., 1987). Urinary AFM1 is detectable by high pressure liquid chromatography (HPLC) and correlates with other aflatoxin biomarkers (Groopman et al., 1993), such as circulating aflatoxin covalently bound to albumin (Gan et al., 1988). The ranges of urinary AFM1 detected by HPLC have included 8.0 to 801 pg/ml in Guinea (Polychronaki et al., 2008) and 170 pg/ml to 5.2 ng/ml in Shanghai, China (Qian et al., 1994).

Among the resource-limited settings where aflatoxin exposure is endemic, rigorous and inexpensive methods to detect urinary AFM1 are urgently needed, particularly for population-level studies targeting areas of high

exposure risk. Enzyme-linked immunosorbent assay (ELISA) is an alternative method to detect urinary AFM1 and has been applied to the analysis of human urine and milk (Zhu et al., 1987; el-Nezami et al., 1995). Helica Biosystems produces a rapid immuno-assay kit for detection and quantification of urinary AFM1 (Sabran et al., 2012). The manufacturer has indicated that the kit has yet to be internally validated by HPLC (Thu Huynh, personal communication), and internal performance characteristics were determined using urines to which AFM1 was added (spiked). Furthermore, the Helica ELISA has not received approval from the US Food and Drug Administration, and we are not aware of any published studies that compare the Helica urinary AFM1 ELISA to HPLC.

The purpose of our report is to comparatively present findings of urinary AFM1 that we generated by both Helica's ELISA and HPLC. We measured urinary AFM1 in samples from 52 Haitian patients using the Helica ELISA, but because samples with unquantified trace amounts of AFM1 were prevalent, we followed ELISA with immuno-affinity enrichment coupled to HPLC.

Materials and Methods

Urinary AFM1 ELISA kits were purchased from Helica Biosystems (Santa

Ana, CA, USA). With aliquots of non-Haitian urine, we prepared reference samples containing 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0, 3.0 and 4.0 ng AFM1/ml urine, using a spectrophotometrically verified standard of AFM1 (Trilogy Analytical Laboratory, Inc., Washington, MO, USA). Those reference urines were tested in six replicates¹ within a single assay run to determine the limit of detection and the limit of quantitation. All samples were assayed according to the manufacturer's instructions. A four-parameter logistic calibration curve was generated for each plate with online software (www.readerfit.com) based on absorbance values from urines provided with the kit containing 0.150, 0.400, 0.800, 1.50, and 4.00 ng AFM1/ml. To characterize cross-reactivity with other aflatoxins, aliquots of urine with no detectable AFM1 were spiked with 1.0 ng/ml of AFB1, AFB2, AFG1, or AFG2.

ELISA was used to test 52 urines obtained in Haiti, a country of endemic aflatoxin contamination (Schwartzbord et al., 2013), collected from adult patients recruited at GHESKIO Health Center in Port-au-Prince during July of 2012. Following informed consent, samples (10-30 ml) were kept frozen at -20°C. Approval was received from the GHESKIO internal review board and the Cornell Institutional Review Board for Human Participants

¹ Replication was done after 1:20 dilution of urines. For example, with each single, dilute urine (1:20 dilution with de-ionized water) representing a different spike, 3 replicate draws were applied to three mixing-wells on a micro-titer plate and further diluted (1:3) with assay diluent (Helica Biosystems). Following the second dilution, duplicate draws of 100 µl from each mixing-well were applied to individual wells on anti-body coated micro-titer plate.

(ID#0908000519).

HPLC was used to evaluate our ELISA results from Haitian samples, which had detectable, but low AFM1, and a modified method by Tang *et al.* was used for sample preparation (Tang et al., 2008). Each sample (15 ml) was diluted 1:1 with 4% phosphoric acid. Samples were spiked with 125 pg of AFB2 as an internal standard (Walton et al., 2001) and centrifuged (4°C) for 20 min at 2100 g. The supernatant was passed through a 6 cc-capacity solid-phase extraction HLB² column by Waters (Milford, MA) that had been conditioned with 4 ml methanol, followed by 4 ml de-ionized water. After washing the column with 4 ml of 5% methanol twice, we eluted it with 4 ml of 100% methanol, and the eluate of each sample was diluted with 36 ml deionized water (pH 6.4) and applied to a VICAM AFM1 HPLC immuno-affinity column (Milford, MA). The column was washed with 10 ml deionized water twice and eluted into a silanized glass tube with 2.5 ml of 3:2 acetonitrile:methanol. The eluate was reduced to dryness under high purity nitrogen at 50°C and reconstituted with 200 µl of 3:2 acetonitrile:methanol and 200 µl of de-ionized water. Using an Agilent 1100 Series HPLC (Santa Clara, CA), 50 µl of filtered sample (0.22 µm filter, Fisher Scientific, Waltham, MA) was injected onto a 150 mm reversed phase column held at 40°C (C18, LCTech, Dorfen, Germany). The mobile

² HLB (Hydrophilic-Lipophilic-Balanced) sorbent is water-wettable and reversed-phased.

phase consisted of 24% acetonitrile and 8% methanol in water with a flow rate of 1.0 ml/min. The HPLC was coupled to a fluorescence detector with an excitation wavelength of 360 nm and an emission of 440 nm. For each run, a standard curve was created using duplicate injections of AFM1 in mobile phase that were equivalent to 2.7, 6.7, 130, and 530 pg/ml urine and injections of AFB2 equivalent to 5.3 and 8.5 pg/ml urine. Precision of the HPLC method was determined by assaying urines containing additions of 1000, 33, 20, 13, 7, and 3 pg AFM1/ml urine, in triplicate.

The limit of detection (LOD) for HPLC was set at the lowest quantity of AFM1 injected with a signal:noise ratio of 3:1. To determine the LOD for the Helica ELISA, student t-tests were applied, and the LOD was set at the reference urine at which mean absorbance was significantly different from the control urine ($p < 0.05$). Intra-assay and inter-assay variability were calculated to assign the limit of quantitation (LOQ) for ELISA and HPLC. Statistical tests were conducted using the software JMP 9 (SAS Institute, 2010).

Results

Recoveries and coefficients of variation (CVs) of the ELISA and HPLC are in **Tables 1 and 2**, respectively. CVs of ELISA standards (absorbance values) were consistently less than 15%. We set the ELISA LOD at 0.2 ng/ml

($p=0.0183$, compared to the blank), and the LOQ at 0.4 ng/ml. We observed cross-reactivity between the ELISA antibody and three of the native aflatoxins, because nominal amounts of AFM1 (with standard deviation) were 1.94 (0.409), 1.01 (0.304), 0.994 (0.270), and <0.200 ng/ml in samples spiked with 1.0 ng/ml of AFB1, AFB2, AFG1, and AFG2, respectively. When we tested Haitian samples by ELISA, none had AFM1 greater than the LOQ, though trace quantities of aflatoxins between the LOD and LOQ were detected in 14 samples. The limit of detection by HPLC was 2.5 pg injected onto the reversed phase column, and we achieved acceptable recoveries of AFM1 and inter- and intra-assay variability down to 3 pg/ml in our HPLC spiking study. Because injections were equivalent to 1.9 ml urine, our limit of quantitation in the linear range was 2.7 pg/ml urine by HPLC. Fourteen of the Haitian samples had detectable aflatoxins. Eleven samples had greater AFM1 than the LOQ, in the range of 2.94 to 96.5 pg/ml, with a mean of 29.5 pg/ml and standard error of 10.8 pg/ml. Of the 14 samples that tested positive by ELISA, HPLC analysis detected AFM1 in four samples, of which one had less than the LOQ of HPLC and three had 96.6, 91.9, and 6.77 pg AFM1/ml. Chromatograms of two samples with detectable AFM1 by HPLC are in **Figure 1**.

Table 7.1: Recovery and variation of urinary aflatoxin M1 measurement using the Helica Biosystems ELISA.

AFM1 Spike (ng/ml) ^a	Average Recovery (%)	Coefficient of Variation of Calibrated Results (%) ^b
4.0	108	6
3.0	160	13
2.0	142	26
1.0	117	21
0.5	139	16
0.4	158	25
0.3	151	33
0.2	128	74
0.1	252	32

^a Aliquots of urine, from individuals not ingesting aflatoxins, were spiked with AFM1, which was quantified in replicates of 6.

^b Standard deviation divided by arithmetic mean.

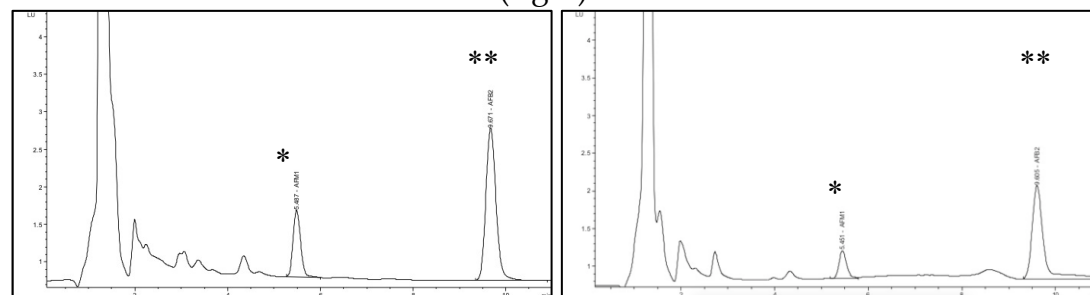
Table 7.2: Recovery and variation of urinary AFM1 measurement using HPLC with fluorescence detection.

AFM1 Spike (pg/ml) ^a	Average Recovery (%)	Coefficient of Variation (%) ^b
1000	98	3
33	104	5
20	104	4
13	103	5
7	128	3
3	158	3

^a Aliquots of urine were spiked, and AFM1 was isolated by immuno-affinity column chromatography, in triplicate, and quantitated.

^b Standard deviation divided by arithmetic mean.

Figure 7.1: Chromatograms from HPLC with fluorescence detection for patients with 91.9 pg AFM1/ml urine (left) and 35.6 pg AFM1/ml urine (right). ^a



^a AFM1 had an average retention time of 5.4 minutes (*), and the internal standard eluted from the HPLC column after 9.3 minutes (**).

Discussion and Conclusions

In the present study, additions of AFM1 in the range of 0.2 to 4.0 ng/ml to reference urine were detected by a commercial ELISA. However, ELISA overestimates increments in urine AFM1 concentration (from 0.4 to 4.0 ng/ml) relative to HPLC analyses of the same urine. The greater estimates of AFM1 concentrations and much higher CVs from ELISA analyses are consistent with cross-reactivity of the ELISA antibody with ligands other than AFM1. We observed a substantial fraction of false positive (with respect to AFM1) ELISA results indicating trace aflatoxin in Haitian samples that, when tested by HPLC, had no detectable AFM1. Furthermore, the ELISA cross reacts with AFB1. Such results suggest that other aflatoxins, aflatoxin metabolites or related ligands were present and detected by ELISA. Despite limitations in specificity of ELISA AFM1 detection in urine at very low concentrations, this ELISA may be useful as a qualitative screen for aflatoxin exposure in

population-level studies that examine the relationship between risk factors and qualitative levels of exposure. Future investigations should reveal the utility of this ELISA at urinary AFM1 above 4 ng/ml.

Unfortunately, there is evidence that lower but chronic concentrations of urinary AFM1 are associated with liver cancer, so precision below 4 ng/ml range would be useful. In a case control study involving 55 liver cancer cases and 267 control cases in China, Qian *et al.* showed that the relative risk of developing liver cancer was 4.4 (95% CI: 2.1 to 9.6) among individuals with detectable AFM1 (>0.170 ng/ml) compared to individuals with no detectable urinary aflatoxin biomarkers (Qian *et al.*, 1994). In a 10-year study, Sun *et al* collected eight urine samples at monthly intervals, recruiting male carriers of hepatitis B virus antigen. Subjects with detectable AFM1 in their urine (>3.6 ng/L) had a 3.3-fold increased risk of developing hepatocellular carcinoma during the study period (Sun *et al.*, 1999).

The Helica ELISA was expressly developed without solid phase extraction (SPE) to optimize ease and low cost to users (Thu Huynh, personal communication) but, perhaps, at the expense of some specificity, accuracy and precision. Other studies have reported stronger correlations between ELISA and HPLC measurement of AFM1 in urine and human milk but used SPE to

remove interfering compounds. Zhu *et al.* noted good agreement between results obtained by HPLC and ELISA with SPE for a selection of 252 urine samples with <0.01 to 3.2 ng AFM1/ml (Zhu *et al.*, 1987). In analysis of human milk, El-Nezami *et al.* used a competitive ELISA preceded by SPE with a limit of detection of 0.005 ng AFM1/ml (el-Nezami *et al.*, 1995). El-Nezami's group reported a correlation of 0.97 between ELISA and HPLC values among detected samples.

In conclusion, we tested 52 Haitian urine samples for AFM1 by the Helica ELISA. Because results ranged between the LOD and LOQ of the ELISA, analysis of the same samples by HPLC followed. A comparison showed that of the 14 samples with detectable aflatoxin by ELISA, four samples had detectable AFM1 by HPLC. Furthermore we demonstrated cross-reactivity between the Helica anti-body and AFB1, and to a lesser extent, AFB2 and AFG1. The limitations of our study should be considered. Our samples had a narrow range of AFM1, and cross-reactivity experiments did not include the dominant metabolites aflatoxin P1 or aflatoxin mercapturic acids.

Nonetheless, this technical brief seeks to inform researchers for whom access to liquid chromatography is limited and an AFM1 ELISA is attractive as an epidemiological tool, especially where chronic exposure to aflatoxin occurs.

Regulatory Approval: The Helica Biosystems ELISA for AFM1 in urine is not approved for diagnostic purposes by the Food and Drug Administration of the USA.

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CHAPTER 8: URINARY AFLATOXIN M1 AMONG ADULTS AND CHILDREN IN PORT-AU-PRINCE AND A RURAL COMMUNITY IN NORTHEAST HAITI

Introduction

Aflatoxins are secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus* which contaminate agricultural commodities, particularly peanuts and maize. Aflatoxin (AF) contamination is most prevalent in hot, humid climates that favor fungal growth and toxin production. Of the major AFs, AFB1 is the most prevalent and toxic. It is designated as a known carcinogen by the International Agency for Research on Cancer (International Agency for Research on Cancer, 2010), and AFs may play a causative role in up to 28.2% of global cases of liver cancer (Liu & Wu, 2010). Furthermore, there is a growing body of evidence from animal models and epidemiological studies implicating AF in immune-dysfunction (Turner et al., 2003). Aflatoxin has been hypothesized to lead to stunted growth in children (Leroy, 2013).

Following ingestion and absorption of AF, hepatic cytochrome P450 mixed function oxidases convert it to several metabolites, of which a few have been validated as biomarkers of exposure. Prominent AF biomarkers include AFM1, which is excreted in human milk and urine and consists of AFB1 with an added hydroxyl moiety (Cheng et al., 1997); AF covalently-bound to circulating blood albumin (Turner et al., 2012); and the adduct AF-N⁷-guanine, a urinary indicator of AF-induced genetic lesions (Groopman et al., 1993). These blood-based and urinary biomarkers, coupled to dietary survey

techniques, have been essential in determining dietary sources of AF exposure (Kensler et al., 2011; Turner et al., 2012).

Several biomarker-based studies offer evidence of within-country variation in AF exposure, due to differences in topography (Peers et al., 1987), food storage practices (Hell et al., 2000), and other post-harvest practices (Turner et al., 2005). Wild *et al.* found that human subjects in Gambia had a higher geometric mean of AF-albumin adducts in rural than peri-urban areas and that exposure was seasonal (Wild et al., 2000). Gong *et al.* showed that regional differences in agroecology and food consumption affected AF exposure in rural areas (Gong et al., 2003). These studies have provided clear evidence that AF exposure is prevalent in tropical and sub-tropical areas where farmers and food processors are unable to prevent contamination, government entities have limited capacity to regulate food safety, and people consume diets based on AF-contaminated foods (Williams et al., 2004).

This combination of a hot, humid climate; limited government and producer resources; and evidence of aflatoxin in the food supply in Haiti motivated this laboratory to assess the prevalence of AF exposure in humans and to better understand the determinants of exposure (Schwartzbord et al., 2013). Haiti is located on the western side of the Caribbean island of Hispaniola and includes a population of 9.9 million people (Central Intelligence Agency, 2014). Previous studies in Haiti demonstrated contamination in maize and peanut butters (Castor et al., 1987; Filbert & Brown, 2012; Schwartzbord & Brown, 2015). Exposure was shown among patients at an urban health center in the capital of Port-au-Prince by measurement of serum AF-lysine (Schwartzbord

et al., 2014). The frequency of peanut consumption in that study population was significantly predictive of detecting AF-lysine, though maize was not.

Prior to the present study, no monitoring of human exposure by measurement of AF biomarkers, to our knowledge, had been conducted with rural Haitian participants. We hypothesized that regional dietary differences within Haiti could cause significant variation of AF exposure in rural versus urban areas. Better nutritional status has been reported among urban Haitians compared to their rural counterparts, due in part to dietary differences between rural areas and urban centers (Smith et al., 2005; Ruel et al., 1999). Exploratory measurement of urinary AF within a subset (n=142) of participants recruited for the present study demonstrated excretion of AFM1 among urban and rural participants (Gerding et al., 2015). Building upon that analysis, the first objective of our study reported here was to measure urinary AFM1 among 367 Haitians and examine dietary AF exposure between an urban and a rural community. The second objective was to test the association between AFM1 and reported dietary intake of potentially contaminated foods, such as maize, peanut products and milk.

Materials and Methods

Study site and design

Our study used a cross-sectional design. Adult males and females seeking medical services at a health center in the capital of Port-au-Prince and the rural town of Quartier Morin (located 8 km from the city of Cap Haitien) were invited to participate. The population of Port-au-Prince is estimated at 2.2

million residents (Central Intelligence Agency, 2014), and the municipality of Quartier Morin has 4,100 residents in town and 22,000 in adjacent villages and hamlets (Institut Haitien de Statistique et d'Informatique, 2012). Subjects were recruited at the Groupe Haitien d'Etude du Sarcome de Kaposi et des Infections Opportunistes Health Center in Port-au-Prince (GHESKIO, n=147) in July 2012 and at the Hôpital Convention Baptiste d'Haïti in Quartier Morin (HCBH, n=191) from September 2013 to January 2014. At HCBH, study subjects also included minors (1 to 17.9 years of age, n=28). Age group and gender information for one HCBH patient was not available. This study was reviewed by and received approval from the Cornell Institutional Review Board for Human Participants (ID#0908000519). Approval in Haiti was received from the GHESKIO internal review board and the Haitian Ministry of Health (MSPP, in French).

Dietary survey and urine sample collection

Intake of dairy, other animal-sourced foods, peanut products, and maize was assessed using a dietary recall survey; participants were asked about the consumption of these foods the day of the survey, the day preceding the survey, and within 8-days preceding the survey. Consumption of non-dairy animal-sourced foods (ASFs) was examined as an index of wealth and intake of nutrients required for AF detoxification. All responses were recorded on a paper questionnaire and entered into a Microsoft Excel file. Urine samples (15-30 ml) were poured from each collection receptacle to a 50 ml centrifuge tube, labeled, sealed with electrical tape, and kept frozen at -20°C. Samples were transported frozen to the Cornell University Nutritional Toxicology Laboratory for analysis.

Chemical Analysis

Urinary creatinine concentration was determined spectrophotometrically using a Jaffee reaction-based kit (Cayman Chemical). For immuno-affinity purification coupled to HPLC analysis of AFM1, 52 individual urine samples from GHESKIO were initially assayed as previously described (Schwartzbord, Severe, & Brown, *under review*). For the remaining samples an aliquot of 10.0 ml urine was immuno-purified and detected using that same method. Briefly, urine was diluted 1:2 with 4% phosphoric acid. Aflatoxin B2 (125 pg) was added as an internal standard (Walton et al., 2001), and urinary aflatoxins were purified by immuno-affinity column chromatography (IAC) (Schwartzbord, Severe, & Brown, *under review*). Sample eluates were dried under high purity nitrogen in silanized glassware and reconstituted in 400 µl of 3:2:5 acetonitrile: methanol: water in preparation for HPLC.

Chromatography was performed on an Agilent 1100 liquid chromatograph coupled to a fluorescence detector, and a 50 µl aliquot of filtered sample was injected onto a 250 mm Agilent XDB-C18 column. Samples were eluted isocratically with a mobile phase of 24:8:68 acetonitrile: methanol: water. Standards were prepared using a spectrophotometrically verified standard obtained from Trilogy Labs (Washington, MO, USA). Each day a standard curve was generated based on 0.0, 5.0, 12.5, 125, and 250 pg AFM1 injected onto the column in duplicate. AFM1 was quantified using Agilent Chemstation software. Intra-day assay variability was determined testing urines with additions of 30, 10, and 5 pg AFM1/ml each in triplicate, and inter-day assay variability at those same concentrations was calculated from

replicate cleanups (see **Appendix Tables to Chapter 8**). Our limits of detection and quantitation were 4.0 pg and 10 pg AFM1/ml urine, respectively.

Statistics

Descriptive statistics were tabulated to summarize age group, gender, detection of urinary AFM1 among participants and reported diet, assessing differences between study sites. Test statistics were considered significant at a p-value of 0.05 or less. Logit models regressed dietary factors to the log odds of AFM1 detection. The distributions of urinary AFM1 on a volume- and creatinine-basis were skewed, so least squares regression was applied to quantitated AFM1 following natural log-transformation to achieve normality of residuals. Separate regressions were performed by the referred period of consumption (i.e., day of, day preceding, or within 8-days of survey), controlling for age, gender and location. All statistical tests were conducted in the code language R, using the program RStudio (RStudio, version 0.98.1091, Boston, MA).

Results

Descriptive statistics of patient diet and independence of categorical variables

Age group, gender, creatinine-adjusted urinary AFM1, and diet of participants are summarized in **Table 1**. Six out of 28 children had detectable AFM1. Of the participants in our study at HCBH, 23% and 77% were male and female, respectively. At GHESKIO, 46% and 54% of patients were male and female, respectively. Creatinine-adjusted AFM1 at the 10%, 25%, 50%, 75%, and 90% quintiles were 2.4, 3.9, 8.4, 39.9, and 140 pg AFM1/mg creatinine, respectively; and on a volume basis, 6.0, 7.2, 11.7, 49.8, and 127 pg AFM1/ml, respectively.

The maximum urinary AFM1 on a volume basis was 700 pg AFM1/ml. Dietary responses were independent of gender and age. **Table 2** highlights results from tests of independence between AFM1 detection and gender, age group, and reported consumption of milk, other ASFs, peanuts, and maize. **Table 2** shows that patients with detectable AFM1 consistently had a higher percent of recalled peanut consumption the day of, the day preceding, or the week prior to sample collection, than those without detectable AFM1.

Regression Analysis

Ordinary least squares estimates, logit estimates and odds ratios are presented in **Table 3**. In our logit model, participant location achieved significance (p-value of 0.014) and gender approached significance (p-value of 0.0554). Probability curves of AFM1 detection were generated using the logit model and are shown against frequency of peanut and maize consumption in **Figure 1**. See **Appendix Tables to Chapter 8** for additional regression results and two-way ANOVA for recalled maize and peanut consumption against quantitated AFM1.

Table 8.1: Age group, gender, creatinine-adjusted urinary AFM1, and diet of participants recruited in urban Port-au-Prince and rural Quartier Morin.

	Port-au-Prince (GHESKIO) ^a	Quartier Morin (HCBH) ^b
Adult, % (<i>n</i>)	100 (147)	87 (191)
Male, % (<i>n</i>)	46 (67)	23 (50)
AFM1		
Detected, % (<i>n</i>) ^c	14 (20)	22 (48)
pg AFM1/mg creatinine (patients with quantifiable AFM1, n=40) ^{d, e}	43.7 ±17.3, 34.5 (3.97-202)	116±38.0, 24.5 (2.44-775)
Dietary intake ^{f, g, h}		
Milk		
Day of survey, % (<i>n</i>)	4 (6)*	10 (23)
Day preceding survey, % (<i>n</i>)	30 (44)	37 (82)
8-days preceding survey, % (<i>n</i>)	67 (98) ***	91 (200)
Mean frequency of consumption, times/week (SEM)	1.6 (0.17) ***	2.8 (0.16)
Other ASFs		
Day of survey, % (<i>n</i>)	33 (49) **	19 (42)
Day preceding survey, % (<i>n</i>)	71 (105)	76 (168)
8-days preceding survey, % (<i>n</i>)	95 (139)	98 (216)
Mean frequency of consumption, times/week (SEM)	3.6 (0.19) ***	5.4 (0.20)
Peanuts		
Day of survey, % (<i>n</i>)	12 (17) **	3 (7)
Day preceding survey, % (<i>n</i>)	16 (23)	23 (50)
Week preceding survey, % (<i>n</i>)	50 (74) **	65 (143)
Mean frequency of consumption, times/week (SEM)	1.1(0.12) **	1.7 (0.13)
Maize		
Day of survey, % (<i>n</i>)	3 (5)	2 (5)
Day preceding survey, % (<i>n</i>)	30 (44) *	19 (41)
8-days preceding survey, % (<i>n</i>)	74 (109) *	62 (135)
Mean frequency of consumption, times/week (SEM)	1.5 (0.12)	1.3 (0.10)

^a Groupe Haitien d'Etude du Sarcome de Kaposi et des Infections Opportunistes.

^b Hôpital Convention Baptiste d'Haït

^c The AFM1 detection limit was 4.0 pg AFM1/ml urine, and the association between location and AFM1 detection approached significance (p-value=0.06).

^d. The limit of quantitation was 10.0 pg AFM1/ml urine.

^e Mean ± standard error, median (range)

^f Chi-square tests were applied to accept or reject independence between patient location and dietary factors.

^g Significance: p-value less than or equal to 0.05 (*), less than or equal to 0.01 (**), less than or equal to 0.001(***)

^h Two-sample t-tests were applied to mean consumption frequency of milk, others ASFs, peanuts, and maize by location.

Table 8.2: Detection of urinary AFM1 across gender, age group, and diet.

		Detection, %(n) ^a		P-value
		No	Yes	
Gender	Male	30 (90)	40 (27)	0.170
Age group	Adult	93 (276)	91 (62)	0.880
Peanuts (day of survey)	Yes	5 (14)	15 (10)	0.005
Peanut (day preceding survey)	Yes	16 (48)	37 (25)	<0.001
Peanuts (8-days preceding survey)	Yes	57 (169)	72 (48)	0.032
Maize (day of survey)	Yes	2 (7)	4 (3)	0.579
Maize (day preceding survey)	Yes	25 (75)	15 (10)	0.105
Maize (8-days preceding survey)	Yes	68 (202)	63 (42)	0.534
Milk (day of survey)	Yes	7 (21)	12 (8)	0.290
Milk (day preceding survey)	Yes	33 (99)	40 (27)	0.372
Milk (8-days preceding survey)	Yes	80 (240)	85 (58)	0.432
Other ASFs (day of survey)	Yes	24 (71)	29 (20)	0.412
Other ASFs (day preceding survey)	Yes	75 (224)	72 (49)	0.739
Other ASFs (8-days preceding survey)	Yes	97 (290)	96 (65)	0.835

^a Reported percentage is within detection sub-level (i.e., of patients without detectable AFM1, 5% consumed peanuts the day of the survey, and of those with detectable AFM1, 15% consumed peanuts the day of the survey.)

Table 8.3: Regression of dietary factors to urinary AFM1 among Haitians, controlling for patient location, age group and gender.

Variable <i>i</i>	Regression of natural log-transformed AFM1		Logit model of AFM1 detection		Odds Ratio (95% CI) ^a
	$B_i \pm \text{standard error}$ ^a	P-value	$B_i \pm \text{standard error}$ ^b	P-value	
Peanuts	1.66± 0.761	0.0366	0.185 ± 0.0728	0.0112	5.35 (2.04-13.9)
Maize	1.96 ± 1.73	0.266	-0.153±0.107	0.152	2.10 (0.393-8.80)
Dairy	-0.236±0.986	0.812	0.0246±0.0632	0.697	1.79 (0.672-4.36)
Non-dairy animal-sourced foods	0.231± 0.600	0.702	-0.114±0.0595	0.0560	1.39 (0.724-2.60)

^{a, b} Variables correspond to recalled nominal consumption of foods the day of urine sample collection and recalled frequency within 8-days preceding collection, respectively.

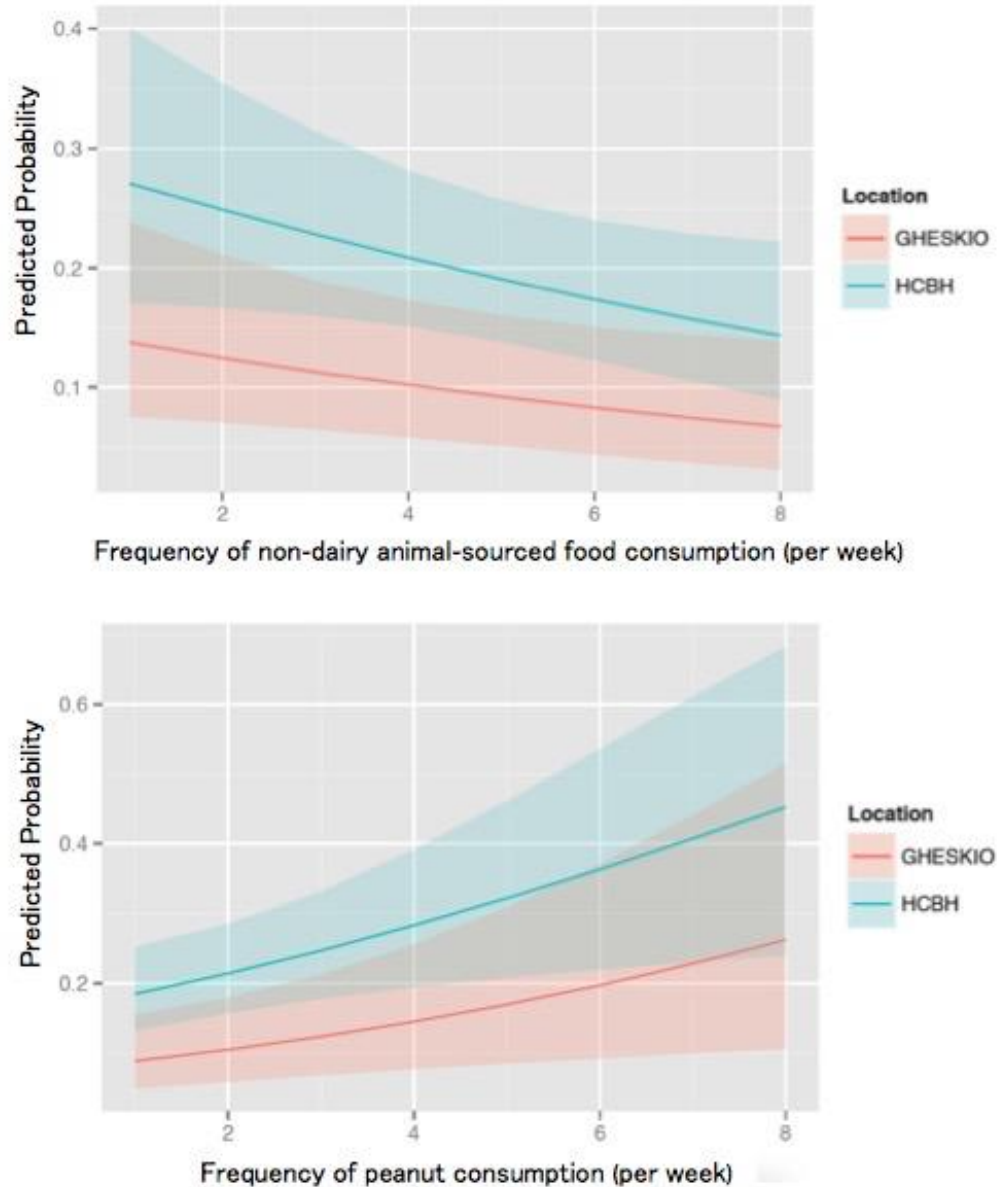
Table 8.4: Ranges and detection rate of urinary AFM1 reported among other populations ^a

Location	Range	Detection rate (total subjects)	Authors
Ashanti region, Ghana	ND-11.6 ng/mg ^b	91% (91)	(Jolly et al., 2006)
Sao Paulo, Brazil	0.002- 0.040 ng/ml	65% (69)	(Romero et al., 2010)
Lower Kindia, Guinea	0.008-0.801 ng/ml	64% (50)	(Polychronaki et al., 2008)
Qidong, China	0.0036-0.243 ng/ml	54% (145)	(Sun et al., 1999)
Nile Delta region, Egypt	0.004-0.508 ng/ml	48% (93)	(Piekkola et al., 2012)
Shanghai, China	0.17-5.2 ng/ml	21% (317)	(Qian et al., 1994)
Nasarawa and Kaduna states, Nigeria	0.05-1.5 ng/ml	14% (120)	(Ezekiel et al., 2014)
Western Cameroon	0.06-4.7 ng/ml	14% (220)	(Njumbe Ediage et al., 2013)
Bangkok, Thailand	0.16-0.55 ng/ml	5% (60)	(Warth et al., 2014)

^a All studies measured AFM1 by either HPLC-fluorescence or LC-MS.

^b Expressed on a creatinine (mg)-basis

Figure 8.1: Predicted probability of urinary AFM1 detection versus frequency of non-dairy animal-sourced foods consumption (top) and peanut products (bottom) among Haitian patients at Port-au-Prince (GHESKIO) and Quartier Morin (HCBH).^{a, b}



^a Probability of AFM1 detection was generated using a logit model regressing recalled frequency of consumption for peanuts, maize, dairy, and non-dairy animal-sourced foods to the log odds of detection, adjusting for patient location, gender, and age group.

^b Shaded regions indicate 95% confidence intervals.

Discussion

The present study sought to assess AF exposure among Haitians in rural and non-rural settings and determine the relationship between urinary AFM1 and intake of various foods, such as peanuts, maize, and dairy. The inclusion of other ASFs in our questionnaire was to provide an index of wealth and diet quality and test whether a better diet, as indicated by ASF intake, might be associated with lower AF exposure. We detected AFM1 in 19% of 367 urine samples collected at HCBH and GHESKIO between 2012 and 2014. Data derived by dietary survey demonstrated that recalled milk, maize, and peanut consumption were significantly associated with location. HCBH patients reported greater milk and peanut consumption, and GHESKIO participants greater maize. The proportions of patients consuming non-dairy ASFs the week of participation at HCBH and GHESKIO were not significantly different, though mean frequency of ASF consumption was greater for HCBH participants. Tests of independence and our logit model showed evidence that peanut consumption was positively associated with detection of the AFM1 biomarker among participants; consumption of milk, other ASFs, and maize were not. Interestingly, frequency of non-dairy ASF approached significance as an inverse predictor of the log odds of AFM1 detection, suggesting that Haitians with access to a higher quality diet were less likely to be exposed to AF.

The association we found in Quartier Morin between AF biomarker detection and peanut consumption is consistent with observations in Port-au-Prince (Schwartzbord et al., 2014), as was the lack of association with maize consumption among patients residing at both sites. Similar to Schwartzbord *et*

al. (2014), we measured a lower maximum concentration of biomarker among samples from Haiti compared to certain countries, such as Ghana and China. Schwartzbord *et al.* (2014) reported 76% detection of AF-lysine, a rate higher than we presently report for urinary AFM1 at GHESKIO (14% detection) and HCBH (22%). That contrast in detection rates for AF-lysine (the proteolytic product of circulating AF-albumin in blood) and urinary AFM1 is consistent with their different half-lives of 2 months and 5-7 days, respectively. (Cheng *et al.*, 1997).

Disparities in urinary AFM1 between patients from HCBH and GHESKIO echo the trend of within-country exposure variation shown in Gambia, Benin and Togo (Wild *et al.*, 2000; Gong *et al.*, 2003). For example, we found that ranges of detectable AFM1 for rural HCBH and predominantly urban GHESKIO patients were 4.51 to 700 and 4.20 to 123 pg/ml, respectively. In comparison, **Table 4** cites proportions of urinary AFM1 detection and minima and maxima reported among other countries of endemic AF exposure. Our results suggest that exposure in Haiti is less elevated compared to Ghana, China, and Cameroon (Jolly *et al.*, 2006; Qian *et al.*, 1994; Sun *et al.*, 1999; Njumbe Ediage *et al.*, 2013). Participants at HCBH excreted urinary AFM1 in ranges approximate to human subjects in rural areas of Nigeria, Guinea, and Egypt (Ezekiel *et al.*, 2014; Polychronaki *et al.*, 2008; Piekola *et al.*, 2012). The prevalence and range of urinary AFM1 among patients in Port-au-Prince approached those found in urban areas of Thailand and Brazil (Warth *et al.*, 2014; Romero *et al.*, 2010).

Special notice is due to the 16% detection of AFM1 among female participants, underscoring potential maternal exposure to AF. Though our study did not enquire female patients whether or not they were pregnant, many were seeking reproductive health services. Myriad investigations elsewhere have established pre- and post-natal AF carry-over from mother to child, and mounting evidence shows that *in utero* and early life exposures to AFs are associated with growth suppression. For example, Wild *et al.* showed a strong correlation between AF-albumin in maternal venous and placental sera and offered evidence of *in utero* exposure (Wild et al., 1991). Shuaib *et al.* measured AF-albumin adducts in maternal blood shortly after delivery and demonstrated that *in utero* exposure was associated with low weight among newborns of participants in Ghana (Shuaib et al., 2010). Circulating AF-albumin among maternal participants during pregnancy has been associated with growth faltering through their newborns' first year of life (Turner et al., 2007). Furthermore women ingesting AF-contaminated foods excrete AFM1 through breast milk, potentiating infant exposure prior to weaning (Zarba et al., 1992), and wholly breastfed children in areas of endemic aflatoxin exposure have been shown to excrete AFM1 (Njumbe Ediage et al., 2013). Certainly, AF exposure among women in Haiti spurs concern.

A few factors limited interpretation of our results. First, patient recruitment periods at GHESKIO and HCBH were neither contemporaneous nor did they occur during the same season. Therefore our finding that AFM1 detection differed significantly between GHESKIO and HCBH patients might not be due to geographic differences in diet and AF contamination *per se*. Seasonality of exposure (Wild et al., 2000) could explain contrasts in urinary AFM1

between GHESKIO and HCBH patients. Second, our inference of exposure risk is largely limited to adults because of our small sample size of youths. Lastly, urinary AFM1 is a robust biomarker of AF exposure, but a weaker index of risk for liver cancer (Qian et al., 1994) and childhood growth faltering (Njumbe Ediage et al., 2013) compared to AF adducts of DNA or blood albumin (Turner et al., 2012). Nonetheless, our findings are supportive of additional biomarker-based risk assessment to evaluate the public health implications of AF exposure in Haiti.

Conclusions

Previously, we established the pressing relevance of AF exposure to broader public health challenges in Haiti, such as liver cancer incidence, stunting prevalence among children, and high burden of infectious disease (Schwartzbord et al., 2013). We presently report compelling evidence of dietary exposure to AFs in Haiti, based on quantification of the biomarker AFM1 in urines obtained from the most representative sampling of rural and urban Haitians to date. Median and maximum urinary AFM1 were approximately 2- and 4-fold greater, respectively, among members enrolled in more rural Quartier Morin compared to those in urban Port-au-Prince. The higher prevalence of urinary AFM1 among patients in the rural HCBH hospital in Quartier Morin is consistent with our hypothesis that AF exposure is greater in rural areas of Haiti compared to its urban centers. Furthermore, recalled consumption of peanuts was the resounding predictor of excreted AFM1. Efforts to control dietary AF exposure in Haiti are paramount and must entail concerted interventions to improve food safety and couple agricultural and food processing improvements with future, population-based

biomarker analysis. Our evidence of elevated AF exposure in the provincial North East of Haiti bolsters support for AF interventions directed to rural areas in particular, where consumption of tainted foods remains a current and insidious threat.

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CHAPTER 9: CONCLUSIONS

Summary of Findings

A review of literature showed evidence that Haitian AF contamination occurs, and the Haitian government has limited resources to attenuate contamination in foodstuffs and prevent human exposure.

The 2011 survey on access to agricultural technologies and AF awareness showed that among the peanut farmers interviewed, 77% were unaware of AF and the threat it poses to food safety. Few of the farmers surveyed disposed of agricultural technologies required to manage AF contamination, illustrating limitations to production and food safety along the Haitian peanut value chain. Food monitoring during 2012 and 2013 showed AF concentration in excess of 20 µg/kg was measured in 94%, 12%, and 5% of peanut butters, snacking peanuts, and maize, respectively. The safety and efficacy of one alternative product, ethanol-rinsed peanut oil, was shown, with demonstrably lower AF than the original contaminated peanuts. In resource-limited contexts such as Haiti, the use of that and other alternative products could have an important role in diverting contaminated foods away from food-insecure populations.

Analysis of blood-based and urinary aflatoxin biomarkers among Haitian patients seeking services at two hospitals in Port-au-Prince and Quartier Morin showed evidence that AF exposure occurs in those communities. Our results underscore that peanut consumption is the most significant and positive predictor of AF exposure among Haitians, though contribution of

maize to exposure cannot be ruled out. Furthermore, our results suggest that exposure to AFs may be greater in rural areas compared to the capital Port-au-Prince.

As Haitian farmers partner with governmental agencies, non-profit organizations, and researchers, scale-up and implementation of alternative uses of contaminated foods and control measures throughout peanut and maize value chains will be key to curbing AF exposure. Future biomarker-based AF exposure studies representative of adult and youth Haitians in urban and rural areas will be key to assessing the broader efficacy of such efforts.

APPENDIX

Appendix Experiment to Chapter 7: Efficacy of solid phase extraction to improve sensitivity of Helica ELISA to AFM1

Materials and Methods

For solid phase extraction (SPE) of human urine samples, an aliquot of urine (3.0 ml) was acidified to pH ~4.5 with 4% phosphoric acid (1-4 drops) and brought to a volume of 6.0 ml with de-ionized water. Each sample was centrifuged for 15 minutes at 2100 g (4°C), after which supernatant (4.0 ml) was applied to a conditioned column with 60 mg of HLB sorbent (Waters). For solid phase extraction (SPE), the column had been conditioned with 2.0 ml of methanol and 2.0 ml of de-ionized water. After passing sample through the column using an electric pump (VICAM) to a flow rate of approximately 1 drop per second, the column was washed with 5% methanol (4.0 ml). Sample was eluted with 1.0 ml methanol into a 1.5-ml vial and stored at -20°C until ELISA analysis. Performance of the SPE with ELISA of AFM1 was examined using urines to which AFM1 (Trilogy Labs) had been added at concentrations of 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.3, and 0.4 ng/ml. Samples with 0.00 to 0.08 ng/ml were extracted and assayed in triplicate.

Authentic AFM1 standard that had been spectrophotometrically verified (Trilogy Labs) was used to prepare working solutions of 0.00, 0.015, 0.030, 0.075, 0.150, 0.400, 0.800, and 1.5 ng AFM1/ml in PBS-Tween (Helica Biosystems). Samples and standards were both diluted 1:10 and had 10% methanol. They were analyzed for AFM1 following the Helica Biosystems protocol. Following generation of a 4-parameter logistic calibration curve

using RStudio (RStudio, version 0.98.1091, Boston, MA), AFM1 values for samples were adjusted accordingly given enrichment by SPE.

Results

The limit of detection was 0.040 ng/ml ($p = 0.0111$, Student's t-test comparing mean raw absorbance to that of blank urine). The limit of quantitation was 0.08 ng/ml.

Table 1: Performance of Helica ELISA following solid phase extraction and quantification of AFM1

AFM1 Addition (ng/ml)	AFM1 Measurement (ng/ml)	Recovery	Coefficient of Variation
0.4	0.350	88%	-
0.3	0.312	104%	-
0.2	0.124	62%	-
0.1	0.094	94%	-
0.08	0.071	94%	21%
0.06	0.061	62%	28%
0.04	0.061	104%	44%
0.02	0.032	88%	43%

Appendix Tables to Chapter 8

Table 8.5: Intra-day recovery and variability of immuno-affinity enrichment and HPLC-fluorometric analysis of urinary AFM1. ^a

Spike	Replicates	AFM1 Average Recovery	Coefficient of Variation
30	3	104%	2.94%
10	3	113%	17%
5	3	146%	24%

^a Urine samples without detectable AFM1 were spiked with known quantities of AFM1, and 10 ml aliquots underwent immuno-affinity enrichment in triplicate within the same day.

Table 8.6: Inter-day recovery and variability of immuno-affinity enrichment and HPLC-fluorescence analysis of urinary AFM1. ^a

Spike	Replicates	Average Recovery	Coefficient of Variation
30	6	108%	15%
10	10	94%	17%
5	12	62%	60%

^a Urine samples without detectable AFM1 were spiked with known quantities of AFM1, and 10 ml aliquots underwent immuno-affinity enrichment in triplicate within the same day.

Table 8.7: Geometric means of AFM1 (pg/ mg creatinine) with 95% confidence interval and one-way ANOVA by dietary groups among Haitian patients.

	Geometric Mean (95% Confidence Interval)		F-Ratio	P-value
	Yes	No		
Milk today?	13.9 ^a	31.6 (18.9-52.8)	0.798	0.378
Milk yesterday?	33.9 (15.8-72.8)	26.7 (13.4-53.3)	0.234	0.631
Milk past 8-days?	29.8 (17.5-50.8)	29.0 (4.2-202)	0.001	0.974
Other ASFs today?	37.5 (17.4-80.6)	27.5 (14.8-51.3)	0.301	0.586
Other ASFs yesterday?	34.0 (18.3-63.2)	20.8 (8.9-48.4)	0.821	0.371
Other ASFs past 8-days?	30.7 (18.5-50.8)	16.3 ^a	0.316	0.577
Peanuts today?	67.7 (22.8-201)	24.2 (13.9-42.1)	3.03	0.089
Peanuts yesterday?	32.9 (14.8-73.1)	27.3 (14.0-53.3)	0.141	0.709
Peanuts past 8-days?	34.0 (19.3-59.9)	15.7 (5.8-42.2)	1.48	0.231
Maize today?	46.9 ^a	29.4 (17.7-48.6)	0.0886	0.768
Maize yesterday?	30.1 (5.6-163)	29.7 (17.3-51.0)	<0.001	0.9858
Maize past 8-days?	34.1 (18.2-63.9)	24.1 (10.1-57.5)	0.482	0.4916

^a Sample size of 3 or less.

Table 8.8: Two-way ANOVA F-tests of quantitated urinary AFM1 (pg/mg creatinine) by recalled peanut and maize consumption among Haitien patients.

Variable	Consumption, day of survey		Consumption, 1-day preceding survey		Consumption, 8-days preceding survey	
	F-statistic	P-value	F-statistic	P-value	F-statistic	P-value
Peanuts	0.115	0.736	0.369	0.547	1.276	0.266
Maize	0.060	0.808	0.231	0.634	0.001	0.975
Interaction	-	-	0.098	0.757	0.005	0.944

Table 8.9: Two-way ANOVA F-tests of quantitated natural log-transformed urinary AFM1 by recalled peanut and maize consumption among Haitien patients.

Variable	Consumption, day of survey		Consumption, 1-day preceding survey		Consumption, 8-days preceding survey	
	F-statistic	P-value	F-statistic	P-value	F-statistic	P-value
Peanuts	2.969	0.0932	0.139	0.713	1.421	0.241
Maize	0.198	0.6588	0.001	0.975	0.253	0.618
Interaction	-	-	1.41	0.243	0.161	0.690

Table 8.10: Regression of dietary consumption variables to quantitated, log-transformed urinary AFM1, controlling for patient location, age group and gender.

	Consumption, 1-day preceding survey		Consumption, 8-days preceding		Frequency of consumption	
Variable	$\beta \pm$ standard error	P-value	$\beta \pm$ standard error	P-value	$\beta \pm$ standard error	P-value
Peanuts	-0.0526 \pm 0.573	0.927	0.659 \pm 0.738	0.378	0.0369 \pm 0.157	0.815
Maize	0.0769 \pm 0.865	0.929	0.254 \pm 0.579	0.664	-0.0099 \pm 0.192	0.959
Dairy	0.0770 \pm 0.594	0.898	0.110 \pm 1.04	0.917	-0.0937 \pm 0.142	0.514
Non-dairy animal-sourced foods	0.555 \pm 0.641	0.393	0.0793 \pm 1.35	0.953	0.110 \pm 0.115	0.344

Table 8.11: Logit regression of dietary consumption variables to log-odds of detection of urinary AFM1, controlling for patient location, age group and gender.

	Consumption, day of survey		Consumption, 1-day preceding		Consumption, 8-days preceding	
Variable	$\beta \pm$ standard error	P-value	$\beta \pm$ standard error	P-value	$\beta \pm$ standard error	P-value
Peanuts	1.677 \pm 0.484	<0.001	1.08 \pm 0.307	<0.001	0.609 \pm 0.302	0.0438
Maize	0.740 \pm 0.770	0.337	-0.661 \pm 0.383	0.0838	-0.153 \pm 0.289	0.597
Dairy	0.582 \pm 0.472	0.218	0.257 \pm 0.293	0.381	0.373 \pm 0.414	0.368
Non-dairy animal-sourced foods	0.330 \pm 0.325	0.310	-0.320 \pm 0.318	0.315	-0.632 \pm 0.719	0.379

Appendix to Methods of Chapter 4: Peanut Farmer Livelihood Survey Questionnaire

Note: This questionnaire was originally prepared in Haitian Kreyol for farmer interviews.

Informed Consent Completed ☐ (Do not begin survey until informed consent is completed)

Date of interview [day/month/year] ____ / ____ / ____

Interviewer Name: _____

Respondent name: _____ ☐ Female ☐ Male

Age of Respondent: _____

Livelihoods

1. Address of household or zone in which the farmer lives

2. Including yourself, how many people live in this household?

_____ total # of people who live in the home

_____ # of these people who are direct family members

3. Of these, how many are children?

_____ total # of children who live in the home

_____ # of children these children who are direct children of
the farmer

4. For each of your children, please state if they are a boy or a girl followed by their age, their level of education and the cost of their education if applicable.

#	Male or Female	Age	Level of education	Cost of education per year
1				Gourde
2				Gourde

3				Gourde
4				Gourde
5				Gourde
6				Gourde
7				Gourde
8				Gourde
9				Gourde

5. If your children do not attend school, please explain why.

6. What is the final level of education you have completed?

1. I didn't go to school
2. Primary
3. Secondary
4. Professional school
5. University
6. Other _____

7. What other sources of income do you have?

1. I don't have other forms of work
2. Construction
3. Merchant
4. Chauffeur
5. Teacher
6. Moto taxi driver
7. I work for an organization _____ (name of the org)
8. Other _____

8. Please list all sources of income for each family member in your household.

Household member	Farmer	Construction	Merchant	Chauffeur	Teacher	Moto Taxi	Some Org.	Other

Instructions: Write the role of the family member in box labeled "Household member" such as "spouse" and mark an "x" for all that apply. Record additional information in the space provided below the table.

Additional Information:

9. Do the incomes of the people who live in your home support your household expenditures?

1. Yes
2. No (if no, move to #11)

10. If yes, whose incomes support the household?

11. About how much money do you make per week? _____ Gourde

12. About how much money do you spend on food each week? _____
Gourde

13. Does anyone in your household ever seek medical attention by a trained doctor or nurse?

1. Yes
2. No (if no, move to #15)

14. If yes, about how many times in one year do you seek this attention and about how much money do you spend on this each year?

How many times in one year: _____

How much money is spent each year: _____ Gourde

15. Do you seek any other types of medical attention from anyone other than a professional doctor or a trained nurse (for example, lakay boko)?

1. Yes
2. No (if no, move to #18)

16. If yes, what type? _____

17. If yes, about how many times in one year do you seek this attention and about how much money do you spend on this each year?

Type of medical attention sought: _____

How much money is spent each year: _____ Gourde

18. How much of the food you eat is grown by you or members of your household?

1. All of it
2. Most of it
3. Some of it
4. None of it
5. I don't have a garden

19. If any of the food consumed is self grown, who takes responsibility for growing this food? (Circle all that apply)

1. Not applicable, because we don't grow our own food
2. Everyone
3. My spouse
4. Brother
5. Sister
6. Daughter
7. Son
8. Other _____

20. What expenditures do your family have and about how much money is spent on each of these per month?

Directions: List all expenditures and write the amount of money spent each year.

expenditure _____
Gourde

money spent each year _____

expenditure _____
Gourde

money spent each year _____

expenditure _____
Gourde

money spent each year _____

expenditure _____ money spent each year _____
Gourde

expenditure _____ money spent each year _____
Gourde

expenditure _____ money spent each year _____
Gourde

expenditure _____ money spent each year _____
Gourde

expenditure _____ money spent each year _____
Gourde

OR estimate amount for all expenses _____ Gourde

21. For how long have you been a farmer? _____
22. Do you grow any crops other than peanuts?
1. Yes
 2. No (If no, go to question 24)
23. If yes, what other crops do you grow? (Circle all that apply and fill in responses as necessary)

Maniok

How many times do you harvest maniok each year? _____

How much maniok do you plant each year? _____

How many sacks of maniok do you harvest each harvest?

(1 - 5 saks) (6 - 10 saks) (11 - 15 saks) (16- 20 saks) Lot

_____ (gourde)

Rice

How many times do you harvest rice each year? _____

How much rice do you plant each harvest? _____ (marmite) or _____ (bol)

How much rice do you harvest each harvest? _____ (marmite) or _____ (bol)

How much money do you make each harvest? _____

Sugar Cane

How many times do you harvest sugar can each year? _____

How much sugar cane do you plant each year? _____

How many dozen do you harvest each year? _____

How much money do you make each year? _____

Corn

How many times do you harvest each year? _____

How much corn do you plant each harvest? _____ (marmite)

How many marmites of rice do you harvest each time you harvest? _____
(marmite)

How much money do you make each harvest? _____

Banana

How many times do you harvest banana each year? _____

How many banana do you plant each year? _____

How many rejim do you harvest each year? _____

How much money do you make each harvest? _____

Pwa

How many times do you harvest pwa each year? _____

How much pwa do you plant each harvest? _____

How many marmites do you harvest each time you harvest? _____ (marmite)

How much money do you make each harvest? _____

What type of pwa do you plant? _____

24. Do you raise any livestock?

1. Yes
2. No (If no, skip to question 31)

25. If yes, what kind?

1. Cow
2. Goat
3. Pig
4. Volay
5. Horse or Donkey
6. Other _____

26. Have you ever purchased feed for your livestock? (If no, skip to question 29)

1. Yes
2. No (if no, move to #29)

27. What kind of feed have you purchased? _____

28. How much did you pay for this livestock feed? _____ Gourde

29. Do you receive any technical veterinary services?

1. Yes

2. No

30. From whom have you received veterinary services? -

31. How much land do farm and what rights to you have to that land?

Type of Property	Peanuts	All Other Crops	Total
Land I own			
Land I rent			
Sharecrop			
Shared family land			

Peanut Production

32. For how long have you been farming peanuts? _____

33. What type of peanuts do you grow?

1. 3 month (Valencia/Spanish variety)
2. 5 month (Runner variety)
3. Both 3 and 5 month varieties
4. Other _____

34. Would you be interested in testing other types/varieties of peanut seed?

1. Yes
2. No

35. Where do you buy your peanut seed? (Circle and fill in all that apply)

1. Market, name _____
2. Organization, name _____
3. From a Cooperative name _____
4. Other _____

36. How much do you pay for peanut seed? (Fill out chart below)

Currency	Prepared (shelled)	Not Prepared (in shell)
Gourde		

37. Does the price of peanut seed change?

1. Yes
2. No (if no, move to #39)

38. If yes, what is the most you have paid and what is the least you have paid?

Price	Prepared (shelled)	Not Prepared (in shell)
Highest Price in Gourde		
Lowest Price in Gourde		

39. What do you do to prepare your land before you plant peanuts?

1. Use a tractor
2. Hire people to help
3. I do it on my own
4. Other

40. How much money do you spend to prepare your land to plant peanuts?

_____ Gourde

41. How many days do you need to prepare the lane for planting peanuts?

42. Do you hire people to plant peanuts for you?

1. Yes
2. No

43. How many times do you weed peanuts? _____

44. Do you hire people to weed peanuts?

1. Wi
2. Non (If no, move to 50)

45. If yes, how many people do you pay each day? _____ # people per day

46. If yes, how much do you pay each person per day _____ Gd/H\$ per person per day

47. How many days does it take to weed all of your peanuts? _____

48. If you pay people to help you, do you provide them with food?

1. Yes

2. No
49. If you give them food, how much does this cost you each day? _____ Gourde
50. When it is time to harvest, do you pay people to help you?
 1. Yes
 2. No (If no, move to 56)
51. If yes, how many people to you pay each day? _____ # people per day
52. If you do hire help, how much do you pay each worker per day?
 _____ gourde per worker/per day
53. How many days does it take until you finish harvesting your peanuts?
54. If you do hire help, do you provide food and/or drink to your workers?
 1. Yes
 2. No
55. If yes, how much does this cost each day? _____ Gourde
56. About how many marmites of peanuts do you harvest each time you harvest?
 _____ mamit pa rekolt
57. Do you sell the peanuts that are harvested on your land?
 1. Yes
 2. No (If no skip to question #61)
58. If yes, where do you sell them?
 1. In the market
 2. To a cooperative _____ (name of coop)
 3. Other _____
59. If yes, what is the highest price and what is the lowest price at which you sell
 Pi wo pri _____
 Pi piti pri _____
60. If price varies, explain why.

61. What tools do you use to farm peanuts and do you own, rent, or share them?

Tools	Do not use	Own	Rent	Share/Borrow
Machete				
Rake				
Hoe				
Pick				
Traction Animal				
Tractor				
Irrigation				
Pesticide				
Lot _____				

62. Of the tools listed above, which one do you think is most important for planting peanuts?

1. Machete
2. Rake
3. Hoe
4. Pickax
5. Ox plow
6. Tractor
7. Irrigation
8. Agrichemicals
9. Other _____

63. If you do use pesticides and chemicals, what type do you use?

Instructions: If they know the name of the chemical they are using, please write it.

1. Fungicide _____
2. Herbicide _____
3. Insecticide _____
4. Fertilizer _____
5. Other _____

64. If you do use any of the pesticides and chemicals listed above, what do you use each of these pesticides for?

1. Insects _____
2. Mold _____
3. Disease _____
4. Weeds _____
5. Other _____

Impact

65. Since you began planting peanuts, have you seen a change in the culture of farming?

1. Yes
2. No (If no, go to question 67)

66. If yes, what changes have you observed? For example, what has changed for the better and what has changed for the worse?

67. Have you attended trainings on how to plant peanuts?

1. Yes
2. No (If no, skip to question # 72)

68. If yes, what did you learn?

69. Did you share this information with other farmers?

1. Yes
2. No

70. Would you like to receive more training on peanut farming?

1. Yes
2. No

71. If yes, is there anything in particular that you would like to learn in these trainings?

72. If you have not attended a peanut farming training, are you interested in doing so?

1. Yes
2. No (if no, move to #74)

73. If yes, what would you like to learn?

74. If no, why not?

75. Do you want to increase the amount of peanuts you produce each time you harvest?

1. Yes
2. No

76. What do you think needs to happen to increase your levels of peanut production?

Instructions: Give them a chance to speak spontaneously and to prioritize. If they don't have ideas, offer ideas by prompting with topics such as finances, seed varieties, chemicals, tools and equipment...)

77. Are you familiar with MFK or Meds and Food For Kids?

1. Yes
2. No (if no, move to #79)

78. If so, what do you know about Meds & Food For Kids?

79. Are you familiar with peanut butter that treats malnutrition such as Medika Mamba or Plumpynut?

1. Yes
2. No (if no, move to #81)

80. If so, what do you know about it?

81. Are you familiar with Gregory Antènor?

1. Yes
2. No (if no, move to question 83)

82. If so, what do you know about him?

83. Are you familiar with Jamie Rhoads?

1. Yes
2. No (if no, go to question 85)

84. If so, what do you know about him?

85. Are you familiar with demonstration plots for best practices?

1. Yes
2. No (if no, go to question 90)

86. If so, what do you know about them?

87. Have you seen any of these plots?

1. Yes
2. No

88. If yes, where was the plot located that you visited? _____ (Name of zone)

89. If yes, what do you remember about this plot and what did you learn?

90. If you have not visited a demonstration plot, would you be interested in doing so?
1. Yes
 2. No
91. Are you familiar with fungicide?
1. Yes
 2. No
92. Have you ever tried using fungicide?
1. Yes
 2. No (If no, move to #95)
93. If yes, are you currently using it?
1. Yes
 2. No
94. If you tried fungicide, what did you like about it and what did you not like about it?
-
-
-
95. If you have never tried it, why not?
-
-
-
96. If you wanted to use fungicide, would you be able to find it?
1. Yes
 2. No
97. Based upon what you know about fungicide right now, would you be interested in buying it?
98. Are you aware that fungicides have safety labels with important information?
1. Yes
 2. No
99. Are you aware that special clothing is required to apply fungicides?
1. Yes
 2. No (if no, move to question #101)
100. If yes, do you wear protective clothing?

1. Yes _____ (include what type of clothing they wear)
 2. No
 3. Not applicable, because I do not use fungicide
101. Are you familiar with aflatoxin in peanuts?
1. Yes
 2. No (if no, move to #103)
102. If yes, what do you know about aflatoxin?
- _____
- _____
103. Do you currently work with anyone or any organizations to improve your peanut farming?
1. Yes
 2. No (if no, move to #106)
104. If yes, who? _____ Name of person/organization
105. If yes, can you tell me about your relationship with this person/organization that you work with to improve your peanut farming and what activities you do with them?
- _____
- _____
- _____
106. Do you ever face difficulties selling your peanuts?
1. Yes
 2. No (if non, move to #108)
107. If so, what type of difficulties do you face?
- _____
- _____
- _____
108. Do you sort your peanuts?
1. Yes
 2. No (if no, move to #110)
109. If so, into what groups do you separate them? _____
110. Do you or any family members consume any of the peanuts that you grow?

1. Yes
2. No

111. Do any of the children eat the peanuts?

1. Yes
2. No

112. If yes, what are their ages?

1. Age of child #1 _____
2. Age of child #2 _____
3. Age of child #3 _____
4. Age of child #4 _____
5. Age of child #5 _____
6. Age of child #6 _____
7. Age of child #7 _____
8. Age of child #8 _____
9. Age of child #9 _____

113. About how many peanuts does your household consume? _____ (# of marmites per year)

I am finished with the survey. Do you have any additional information that I did not ask you about that you would like to share with me before I leave? (Please be sure to record responses).
